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# THE JOURNAL OF BIOLOGICAL CHEMISTRY

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VOLUME XXXVI

BALTIMORE

1918

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

PUBLISHED BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH FOR THE  
JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

WAVERLY PRESS  
THE WILLIAMS & WILKINS COMPANY  
BALTIMORE, U. S. A

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## CORRECTION.

On page 76, Vol. XXXVI, No. 1, October, 1918, under column 3 of formula, for *d-epichitosaminic* read *d-epichondrosaminic*; under column for *d-chitosaminic* read *d-chondrosaminic*.

## CORRECTION.

On page 97, Vol. XXXVI, No. 1, October, 1918, line 13 of the second paragraph, following the parenthesis insert 25 cc. of 20 per cent sodium carbonate.



Owing to the difficulties placed in the way of the printing trades by war conditions, *The Journal of Biological Chemistry* desires to cooperate with the printer by eliminating unnecessary labor in the setting up of manuscripts. To accomplish this object, parts of the papers to be printed in different styles of type should be separated in the manuscript. For example, every experiment, table, or quotation of over five lines should begin on a new sheet. When the text is resumed, a fresh sheet should be started. Separate sheets should be used for the running head-lines, title, foot-notes, and bibliography. The name of the author and the laboratory where the work was done together with the words, "(Received for publication, \_\_\_\_\_, 1918)," should also be written on a separate sheet.

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## STUDIES IN URIC ACID METABOLISM.

### I. THE INFLUENCE OF HIGH PROTEIN DIETS ON THE ENDOGENOUS URIC ACID ELIMINATION.

BY HOWARD B. LEWIS AND EDWARD A. DOISY.

(*From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.*)

(Received for publication, August 10, 1918.)

That ingestion of large amounts of protein food free from purines may markedly increase the endogenous output of uric acid is now generally accepted as a result of the work of many investigators (1, 2, 3, 4, 5). The cause of this increase is, however, still a subject of controversy. The most generally accepted theory, proposed by Mareš (6) and supported by the work of Mendel and Stehle (5) and others (7, 8), holds that the influence exerted by foodstuffs, particularly protein, on the endogenous uric acid excretion is due to their activity in stimulating the glands of the gastrointestinal tract to secretion. The increased elimination of uric acid may also be regarded as due to the excessive work of body cells in general in the metabolism of the unusual amounts of amino-acids formed from digestion of protein. Taylor and Rose (4) have also suggested that since nucleic acid must be synthesized from the units of the protein molecule, when large excesses of protein digestion products are presented to the cells, nuclear anabolism and catabolism are exaggerated, an application of the law of mass action. If it can be shown that any amino-acid or group of amino-acids takes part in purine synthesis from non-nuclear material, then ingestion of proteins rich in such amino-acids might be expected to stimulate the nuclear metabolism more than protein food poor in amino-acids which contribute to purine anabolism.

Ackroyd and Hopkins (9) have recently discussed the formation of purine bases from amino-acid precursors and have presented

evidence to show that histidine and arginine may function in purine synthesis in the rat. Rats were kept on a diet from which these two amino-acids were excluded and the elimination of allantoin, which is in this species the end-product of purine metabolism, was followed. When both arginine and histidine were removed from the diet the amount of allantoin excreted was decreased, but returned to normal on addition of these acids to the diet. Previous experiments with histidine fed to dogs (10, 11) had shown no changes in the allantoin excretion, but Ackroyd and Hopkins consider that the experimental conditions of previous investigators were unfavorable, inasmuch as histidine was added to an already adequate dietary, in which the current needs of the body for purine precursors were already supplied. No investigation of the influence of the amino-acids, histidine and arginine, on the uric acid excretion in man appears to have been made.

In the present series of experiments, a comparison of the effects on uric excretion in man of high protein diets, rich and poor in their content of these diamino-acids respectively, has been made, in the belief that, if the increased purine output after high protein diets is due to increased purine synthesis from arginine and histidine, a high protein diet rich in these amino-acids might cause more marked rises in uric acid elimination than a similar diet poor in these amino-acids.

The subjects were two healthy men who performed the usual routine laboratory work during the course of the investigation. Uric acid was determined by the Benedict-Hitchcock modification of the Folin-Denis colorimetric method, creatinine by the micro-colorimetric method of Folin, and total nitrogen by the method of Kjeldahl. Creatinine was determined as a control on the complete collection of the urine.

During the fore periods (low protein), the diet consisted mainly of potatoes with a small amount of bread. In the first experimental period (high nitrogen, low arginine and histidine), the main source of the protein of the diet was glidine, a commercial diabetic food prepared from wheat. This material, which contained 15.1 per cent nitrogen, was made into muffins and also sprinkled on the other articles of food. From 250 to 300 gm. were consumed daily. In the high arginine-histidine period it was planned to supply the bulk of the protein of the diet by peanuts from which the endo-

sperm had been removed before eating. Due to some digestive difficulties particularly with Subject D, it was impossible to make peanuts the sole source of protein of the diet, and cottage cheese, eggs, and gelatin, all of which are relatively high in their histidine and arginine content, were added to the diet. The lack of utilization of the proteins of the nuts may account for the lower nitrogen during these periods.

Accurate data for the histidine and arginine content of proteins are available only in a few instances, the most accurate being those obtained by the Van Slyke method for the determination of characteristic chemical groups. The more recent analytical results obtained by this method for certain of the proteins fed have been collected in Table I. It will be noted that the arginine-histidine

TABLE I.

*Histidine and Arginine Nitrogen in Percentage of Total Nitrogen of Proteins.*

Protein.	Arginine-nitrogen.	Histidine-nitrogen.
	<i>per cent</i>	<i>per cent</i>
Gliadin* (wheat) . . . . .	5.45	3.39
Casein† . . . . .	9.31	6.55
Gelatin‡ . . . . .	14.70	4.48
Arachin§ (peanut) . . . . .	23.77	2.78
Conarachin§ (peanut) . . . . .	25.78	2.72

\* Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

† Crowther, C., and Raistrick, H., *Biochem. J.*, 1916, x, 434.

‡ Van Slyke, D. D., *J. Biol. Chem.*, 1911-12, x, 15.

§ Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1917, xxx, 33.

content of gliadin is about one-third that of the proteins of the peanut and about one-half that of gelatin and casein. Glutenin and gliadin are present in wheat gluten in approximately equal amounts. No figures for the analysis of glutenin by the Van Slyke method were available in the literature, but results of analysis by the older method of direct isolation of the dibasic amino-acids indicate that it is lower in its arginine-histidine content than the proteins already considered although slightly higher than

gliadin.<sup>1</sup> Glidine,<sup>2</sup> which is to be regarded as a mixture of equal parts of gliadin and glutenin, should thus be distinctly lower in its arginine-histidine content than are casein, gelatin, vitellin, and the proteins of the peanut.

The results of the individual experiments are given in Tables II and III and require little comment. The ingestion of a purine-free high protein diet rich in arginine and histidine gave rise to no greater increase in the excretion of uric acid than did a similar high protein diet low in its content of these amino-acids. If there was, in the presence of large amounts of protein digestion products, any exaggerated anabolism and catabolism of nucleoprotein, in which arginine and histidine played a rôle, this was not evident under the experimental conditions which obtained in the present series.

In view of the recent studies of Denis and Minot (12), in which creatinuria was induced in women by high protein diets, the urine was examined daily for creatine throughout the course of the above experiments. In accordance with the findings of Denis and Minot in men and more recently of Rose and collaborators (13), no creatine was excreted although the nitrogen output exceeded 35 gm. in some instances.

<sup>1</sup> Osborne and associates (*Am. J. Physiol.*, 1906-07, xvii, 231; *J. Biol. Chem.*, 1915, xxii, 259) have obtained the following figures for the arginine and histidine content of wheat glutenin and gliadin respectively: arginine, 4.7 and 3.0 per cent; histidine, 2.2 and 1.8 per cent. Kossel and Kutscher reported analyses of wheat gluten as follows: arginine, 4.4 per cent; histidine, 1.2 per cent. With these compare the percentages of arginine and histidine, of the proteins of the peanut calculated from the figures obtained by the nitrogen distribution method of Van Slyke by Johns and Jones (*J. Biol. Chem.*, 1917, xxx, 33): arginine, 13.5 and 14.6 per cent; histidine, 1.9 and 1.8 per cent.

<sup>2</sup> Since the above was written, it has been possible through the kindness of Mr. H. C. Eckstein and Prof. H. S. Grindley, of the Division of Animal Nutrition, to secure analyses of glidine by the Van Slyke method. The results (average of duplicate determinations), expressed in percentages of total nitrogen, are as follows: ammonia, 22.159; melanin, 0.703; arginine, 6.389; histidine, 3.378; cystine, 1.241; lysine, 1.678; monoamino, 55.417; non-amino, 10.799; total, 101.764.

TABLE II.

*Subject E. A. D. Weight 61 Kilos.*

Period.	Day.	Total nitrogen.	Uric acid.	Creatinine.	Diet.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
I	1	5.68	0.470	1.60	Low nitrogen. Potatoes, bread.
	2	5.18	0.501	1.51	
	3	5.53	0.470	1.72	
	4	5.46	0.433	1.61	
Total .....		21.85	1.874		
Average .....		5.46	0.469		
II	5	17.84	0.535	1.66	High nitrogen, low histidine and arginine. Glidine.
	6	24.94	0.487	1.56	
	7	31.57	0.568	1.61	
	8	41.06	0.787	1.62	
Total .....		115.41	2.377		
Average ..		28.85	0.594		
III	9	17.96	0.578	1.57	Same as Period I.
	10	7.87	0.473	1.51	
	11	5.46	0.363	1.55	
	12	4.52	0.375	1.58	
Total .....		35.81	1.789		
Average ..		8.95	0.447		
IV	13	12.82	0.403	1.57	High nitrogen, high histidine and arginine. Peanuts, eggs, gelatin, cottage cheese.
	14	21.17	0.460	1.67	
	15	27.63	0.581	1.58	
	16	25.57	0.569	1.59	
Total .....		87.19	2.013		
Average .....		21.80	0.503		
V	17	15.22	0.567	1.50	Same as Period I.
	18	9.39	0.500	1.60	
	19	4.76	0.488	1.59	
Total .....		29.37	1.555		
Average .....		9.79	0.518		

TABLE III.  
*Subject H. B. L. Weight 80 Kilos.*

Period.	Day.	Total nitrogen.	Uric acid.	Creatinine	Diet.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
I	1	8.05	0.444	1.65	Low nitrogen. Potatoes, bread.
	2	9.21	0.500	1.73	
	3	8.14	0.406	1.81	
	4	7.15	0.439	1.72	
Total .....		32.55	1.789		
Average .....		8.14	0.447		
II	5	21.03	0.663	1.90	High nitrogen, low histidine and arginine. Glidine.
	6	19.64	0.539	1.78	
	7	29.77	0.669	1.78	
	8	36.55	0.650	1.69	
Total .....		106.99	2.521		
Average .....		26.75	0.630		
III	9	18.55	0.528	1.68	Same as Period I.
	10	11.96	0.550	1.76	
	11	12.18	0.534	1.75	
Total .....		42.69	1.612		
Average .....		14.23	0.537		
IV	12	23.79	0.672	1.74	High nitrogen, high histidine and arginine. Peanuts, eggs, gelatin, cottage cheese.
	13	23.94	0.632	1.90	
	14	20.42	0.647	1.72	
	15	25.14	0.592	1.71	
Total .....		93.29	2.543		
Average .....		23.32	0.637		
V	16	12.61	0.592	1.79	Same as Period I.
	17	9.78	0.528	1.80	
	18	8.92	0.507	1.68	
Total .....		31.31	1.627		
Average .....		10.44	0.542		

## SUMMARY.

Normal men were maintained on purine-free high protein diets of high and low content in arginine and histidine. No differences in the excretion of uric acid following the ingestion of these two types of high protein diet were evident. This would indicate that, under the experimental conditions of the present study, arginine and histidine function no more than the other constituents of the protein molecule in the stimulation of the output of endogenous uric acid following ingestion of a high protein diet.

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## STUDIES IN URIC ACID METABOLISM.

### II. PROTEINS AND AMINO-ACIDS AS FACTORS IN THE STIMULATION OF ENDOGENOUS URIC ACID METABOLISM.

By HOWARD B. LEWIS, MAX S. DUNN, AND EDWARD A. DOISY.

(*From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.*)

(Received for publication, August 10, 1918.)

Of the many hypotheses advanced (1) to account for the origin of the endogenous uric acid of the urine, two, those of Burian and Schur and of Mareš, stand out as most satisfactory. Burian and Schur (2) first clearly distinguished between the two sources of the uric acid of the urine: that derived from the food or exogenous fraction, and that originating from tissue metabolism or the endogenous fraction. They held that the endogenous uric acid excretion might vary widely with different individuals, but that for any one individual it was a constant quantity, unaffected by the intake of purine-free food. Burian (3) has concluded from the analyses obtained in his earlier work (4) that only a small part of this endogenous fraction may be derived from the nucleoprotein of cellular origin. Such an origin would in his opinion involve far too extensive a catabolism of nuclear material to be considered as the main source of endogenous uric acid. Uric acid of endogenous origin, according to this view, is formed mainly from the hypoxanthine of the inosinic acid present in muscular tissue.

Mareš (5) considers that purine-free food may exert a marked influence on the endogenous excretion of uric acid. Others, notably Lambling and Dubois (6), Folin (7), Hopkins and Hope (8), Smetánka (9), Taylor and Rose (10), and Mendel and Stehle (11), have also shown that food free from purine precursors, particularly protein food, may markedly raise the level of endogenous uric acid excretion. Mareš (5, 12) has suggested that this influence on the uric acid excretion is the expression of the wear and

tear on the nuclear material of the secretory glands of the gastrointestinal tract in the work of secretion occasioned by the presence of food in the alimentary canal. Mendel and Stehle (11) in support of this hypothesis concluded that their data "offer no obstacle to the assumption that a portion, at least, of the endogenous uric acid may originate from the activity of the alimentary secretory apparatus." They point out however that the theory of Mareš does not necessarily need to account for all the endogenous uric acid excreted, but may at least explain the source of some fraction of it. The experiments which offer most striking evidence of the rôle of the digestive glands were concerned with the influence of atropine and pilocarpine on uric acid excretion. Atropine, an inhibitor of glandular secretory activity, was shown to check the rise in uric acid output which normally followed ingestion protein food. After the administration of pilocarpine, which is known to stimulate secretion, there was a sharp rise in the uric acid output. Mareš (12) has also reported similar results with pilocarpine. Increase in the mechanical work of the digestive tract (administration of bulky foods or laxatives) was not a factor in the stimulation of uric acid output.

The older methods for the determination of uric acid were hardly suited to the determination of small amounts of uric acid, such as are met with in studies of hourly elimination. The colorimetric method of Folin and Denis as modified by Benedict and Hitchcock is, however, very accurate for the determination of small amounts of uric acid in dilute urines. Moreover, the hourly elimination of uric acid in the fasting individual is subject to certain variations, due to a variety of factors (13), whose rôle is not clearly understood, and the proper control of which has frequently not been secured in former experiments.

The purpose of the experiments to be detailed was to study the influence of proteins and protein derivatives, on the endogenous uric acid excretion, with the use of the newer more accurate colorimetric methods for uric acid determination, and with as complete a control of the variable factors as possible. It was believed that a comparison of the influence of proteins with that of their digestive products, amino-acids, might indicate to what extent the work of the digestive glands is concerned with the increase in uric

acid excretion following ingestion of non-purine protein food. The study of the influence of carbohydrate and fat and their derivatives will be considered in a subsequent publication.

#### EXPERIMENTAL.

##### *Methods.*

The subject of the experiment<sup>1</sup> was a healthy young man, 22 years of age, and about 58 kilos in weight. During a period of over 6 months, a meat-free low protein diet, free also from purine-containing beverages, which may be considered as a "purine-free" diet, was consumed with the exception of a few meals during the holidays, at which a small amount of meat was taken. No attempt was made to secure a quantitative uniformity of the diet. On the evening preceding the day of an experiment, a light supper was eaten and no further food was ingested until the completion of the day's experiment, except the substance whose influence on uric acid excretion was to be studied.

Some of the former experiments in which the hourly excretion of uric acid has been studied are open to the criticism that the volumes of urine collected are low, frequently less than 20 cc. In cases of such small volumes the error due to incomplete emptying of the bladder might be large, whereas in larger volumes this source of error would be inconsiderable. In order to insure reasonably large hourly volumes of urine and thus eliminate as far as possible the errors resulting from the inability to remove the urine quantitatively from the bladder, 200 cc. water were ingested hourly throughout the experimental periods. As a check on the errors from this source, creatinine was also determined in the hourly samples.<sup>2</sup> Since creatinine elimination is uninfluenced by

<sup>1</sup> Two other men also served as subjects. Many of the experiments were duplicated and similar results obtained with these other subjects, but inasmuch as the experiments with M. S. D. were more comprehensive and extended over a longer period of time, the data of these experiments alone are presented.

<sup>2</sup> Creatinine determinations were made in all the experiments reported in the tables. In order to condense the analytical data, the figures for creatinine are omitted in many cases except where variations in the volume of urine and in the uric acid excretion might throw doubt on the completeness of the collection of the urine (cf. Table VIII).

diet and tends to maintain a constant level, it was believed that any marked variation from the normal creatinine level in any period would indicate losses due to incomplete collection of the urine.

Uric acid was determined colorimetrically by the Benedict-Hitchcock modification of the Folin-Macallum-Denis method; creatinine by Folin's micro-colorimetric method; and total nitrogen by the method of Kjeldahl. The samples were preserved with chloroform in tightly stoppered bottles, until the analyses could be completed. In the greater number of the experiments the uric acid determinations were made on the experimental day and those of creatinine on the morning of the following day, although in a few cases this was impossible.

#### *Fasting Control Experiments.*

If changes in the excretion of uric acid following ingestion of various substances were to be of any significance, it was necessary to have accurate information as to the degree and kind of variations to be expected normally in the fasting subjects under the experimental conditions outlined above. Control experiments in which no food was ingested throughout the experiment were carried out at frequent intervals in order to make certain that the level of endogenous uric acid metabolism was not altered by the long continued "purine-free" diet. Protocols of three typical experiments of this sort are given in Tables I and II, these particular experiments being chosen from a considerable number as representing the uric acid level at the beginning and toward the end of the series of experiments respectively. In the majority of the experiments there was a tendency toward a diminished uric acid excretion at the end of the morning hours. This is in accord with the observations of previous investigators (11, 13).

On the basis of these control experiments it seemed reasonable to assume that a rise in the uric acid excretion to a level of 25 mg. per hour or above, following ingestion of some substance, resulted from the action of this substance and was not the result of a normal variation. It was not considered that a rise of a few mg. in any one experiment had necessarily any especial significance.

The following routine procedure was adopted for subsequent experiments. The uric acid excretion for the first 2 hours of the day was determined and if the results were comparable with those of the control days<sup>3</sup> the substance, whose effect on the excretion of uric acid was to be studied, was ingested.

TABLE I.

*Experiment 3. Fasting Normal. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	mg.	mg.	mg.
6-7	122	20.7	61.0	386.7
7-8	210	19.9	58.8	425.8
8-9	291	18.5	64.2	416.1
9-10	150	15.0	63.0	355.5
10-11	92	16.5	59.8	345.0
11-12	122	15.5	59.7	339.1
12-1	311	17.1	62.2	441.6

TABLE II.

*Fasting Normals. 200 Cc. of Water per Hour.*

Time	Experiment 2.		Experiment 24.	
	Volume.	Uric acid.	Volume.	Uric acid.
	cc.	mg.	cc.	mg.
6-7	42	18.3	48	17.4
7-8	220	10.3	98	18.4
8-9	283	21.8	65	19.7
9-10	88	14.9	110	16.2
10-11	132	13.2	51	14.3
11-12	59	15.3	241	15.2
12-1	214	14.1	79	17.7

<sup>3</sup> On 4 days in the series of some 40 experimental days the level of uric acid excretion was found to be greatly increased above the usual level and to remain at this level throughout the entire period. No satisfactory explanation of this variation could be found, and because of this, the fore period of 2 hours was determined upon. *In no case where the excretion of uric acid was at the normal level (about 20 mg. per hour) in the earlier hours of the day did it rise abnormally in the later hours.* The level of the early hours of the day was maintained whether it was abnormally high as in the four experiments referred to or at the normal level as in the majority of the experiments.

*Protein.*

It has repeatedly been shown that the excretion of endogenous uric acid is increased by purine-free protein food. The experiments recorded in Tables III, IV, and V are in agreement with previous work. After ingestion of each of three types of protein food, egg white, cottage cheese, and glidine,<sup>4</sup> there occurred a rise

TABLE III.

*Experiment 4. Protein. Egg White. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	mg.	mg.	mg.
6-7	136	21.7	58.6	484.1
7-8	273	21.8	57.3	455.4
8-9*	245	23.5	58.8	443.4
9-10	244	24.4	58.5	470.6
10-11	166	28.2	58.1	489.7
11-12	75	29.2	57.7	453.0
12-1	332	19.9	59.2	574.3

\* 300 gm. of egg white, poached, eaten at 8 a.m. N content = 5.85 gm.

TABLE IV.

*Experiment 12. Protein. Cottage Cheese. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	mg.	mg.	mg.
6-7	156	19.3	56.2	429.0
7-8	207	19.8	59.2	459.5
8-9	303	20.0	58.5	478.7
9-10*	58	15.2	55.7	281.5
10-11	96	24.1	62.4	579.8
11-12	96	23.2	58.5	595.2
12-1	200	18.0	56.0	628.0

\* 200 gm. of cottage cheese taken at 9.15 a.m. N content = 4.8 gm. (approximately).

<sup>4</sup> Glidine is a commercial "pure vegetable protein food, prepared wholly from wheat." The preparation used in these experiments contained 15.1 per cent nitrogen. According to the analyses of Street (*Connecticut Agric. Exp. Station., Ann. Rep., 1913, 25*) glidine contains no starch and 91.4 per cent protein.

in uric acid excretion clearly above the basal level, a rise which usually reached its maximum during the 3rd or 4th hour after administration of the protein. The two experiments with glidine (Table V) show some sort of a quantitative relationship, for in Experiment 32, 66.6 gm. of glidine increased the excretion more markedly than did half the amount, as in Experiment 31. It was not possible to continue Experiment 32 long enough to determine whether the maximum effect had been reached. No clearly defined differences between the three types of proteins in their action on the uric acid output could be observed.

TABLE V.  
*Protein. Glidine. 200 Cc. of Water per Hour.*

Time.	Experiment 31.		Experiment 32.	
	Volume.	Uric acid.	Volume.	Uric acid.
	<i>cc.</i>	<i>mg.</i>	<i>cc.</i>	<i>mg.</i>
6-7	42	14.1		
7-8	218	17.1	51	17.5
8-9	265*	16.6	245	19.2
9-10	170	23.6	268†	19.8
10-11	208	22.2	156	27.9
11-12	200	16.6	99	26.7
12-1	53	16.1	70	29.2

\* 31.7 gm. of glidine taken dry at 8.00 a.m. N content = 4.8 gm.

† 66.6 " " " " " " 9.00 " N " = 10.1 "

#### *Amino-Acids. Alanine and Glycocoll.*

In order to test the validity of the theory that increases in the excretion of endogenous uric acid after ingestion of protein result from the secretory activity of the glandular tissue of the alimentary canal, occasioned by the work of digestion, the influence of amino-acids, the ultimate cleavage products of protein in the tract, was studied. The results of two typical experiments with alanine and glycocoll are shown in Table VI. It is evident from the results of these and a number of other experiments of the same kind that glycocoll and alanine, amino-acids representing the final products of the digestion of proteins, stimulate the endogenous uric acid metabolism in a manner similar to the proteins themselves,



although the effect is produced more rapidly and is more marked. Thus in Experiment 10 (Table VI), glycocoll equivalent to 1.94 gm. of nitrogen caused an increased uric acid excretion the 2nd hour after ingestion of much the same order of magnitude as that produced by egg white equivalent to three times the above amount of nitrogen, 5.85 gm., during the 3rd and 4th hours after ingestion. The effect of the amino-acid is manifested sooner than

TABLE VI.

*Amino-Acids. Glycocoll and Alanine. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.
Experiment 10. Glycocoll.			
	cc.	mg.	mg.
6-7	150	21.6	56.4
7-8	300	21.9	58.8
8-9*	117	23.4	57.3
9-10	294	30.2	57.9
10-11	210	21.2	55.8
11-12	65	19.7	53.3
12-1	166	19.4	59.7
Experiment 23. Alanine.			
6-7	100	18.9	57.0
7-8	271	17.6	56.4
8-9†	108	21.5	58.8
9-10	291	25.9	55.3
10-11	79	17.1	54.9
11-12	57	17.8	54.2
12-1	36	14.4	47.8

\* 10.4 gm. of glycocoll eaten at 8.00 a.m. N content = 1.94 gm.

† 12.1 " " alanine " " 8.00 " N " = 1.90 "

in the case of the proteins, the excretion of uric acid reaching its maximum the 2nd hour after ingestion and as a rule returning to normal the following hour.

It seemed possible that the effects observed might be due to a *stimulation of the processes of elimination* of uric acid under the influence of the amino-acids, rather than to *increased uric acid formation*; that they might be the result of the removal of pre-formed uric acid and uric acid precursors from the tissues, an

exaggerated elimination of purine reserve stored. If this were the case, ingestion of a second dose of amino-acid after the effect of the first had reached its maximum should not further affect the uric acid elimination, since the increased elimination as a result of the first dose should have caused the removal of any excess present in the system. In Table VII are presented the results of such an experiment, the influence of the administration of successive doses of glycocoll on the same experimental day. The figures clearly demonstrate that the effects of amino-acids on uric acid excretion are not the result of stimulation of excretory processes leading to a

TABLE VII.

*Experiment 10. Amino-Acids. Successive Doses of Glycocoll. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	mg.	mg.	mg.
6-7	80	19.1	58.4	401.6
7-8	229	21.5	57.7	403.0
8-9*	67	24.6	59.5	300.1
9-10	244	30.7	61.0	460.7
10-11	245	20.3	59.8	421.4
11-12†	86	19.1	55.5	360.3
12-1	256	30.2	62.4	524.8
1-2	326	18.3	55.1	475.9
2-3	76	14.7	49.9	395.2
3-4	49	14.1	54.4	345.9

\* 10.2 gm. of glycocoll at 8 a.m. N content = 1.90 gm.

† 10.2 " " " " 11.05 a.m.

removal of preformed uric acid from the body since the administration of a second dose of glycocoll gives rise to an increased uric acid excretion comparable in all respects to the increase produced by a single dose.

#### *Dicarboxylic Amino-Acids. Glutaminic and Aspartic Acids.*

The work of Lusk (14) has clearly proven that the phenomena of the specific dynamic action of protein are associated with the amino-acids or their cleavage products (15). It seemed probable that the increased elimination of uric acid, presumably the result of a stimulation of the metabolism of the nuclear material

of the cell, might be associated with these phenomena of increased heat production under the influence of the amino-acids. According to Lusk no increased heat production follows ingestion of the dicarboxylic amino-acid, glutaminic acid (and presumably also its analogue, aspartic acid). Grafe more recently has maintained that these two amino-acids do exert a specific dynamic action (16), as do also ammonium chloride and several other substances. In view of these considerations it seemed of interest to observe the influence of the dicarboxylic amino-acids on the uric acid metabolism.

TABLE VIII.

*Experiment 11. Dicarboxylic Amino-Acids. Glutaminic Acid. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	mg.	mg.	mg.
6-7	100	18.8	55.5	368.0
7-8	293	19.1	50.9	383.6
8-9*	44	27.8	54.1	219.1
9-10	40	44.4	54.0	256.0
10-11	71	46.8	54.6	394.0
11-12	527	23.7	49.7	548.0
12-1	165	19.9	47.8	396.0

\* 20 gm. of glutaminic acid (N = 1.9 gm.) taken at 8.00 a.m. Solution was facilitated by heat and the addition of 7 gm. of  $\text{Na}_2\text{CO}_3$ . The solution obtained still retained an acid reaction to litmus. After 30 minutes general dizziness and nausea without vomiting, which continued for 4 to 5 hours, and increased with ingestion of water at each hourly period.

Ingestion of glutaminic acid (Table VIII) resulted in an increased excretion of uric acid, more marked than in the previous experiments with glycocoll and alanine. The effect was more prolonged as the normal level was not reached until the 5th hour after ingestion of the amino-acid. The effects on the volume of urine and on the nitrogen elimination were also noteworthy. During the 3 hours of maximum elimination of uric acid the urinary volume was very low, falling below 50 cc. during two periods. This was followed by a marked diuresis with the extraordinary hourly volume of 527 cc. the 4th hour when the uric acid excretion had returned to normal. That these low volumes of urine

were not the result of incomplete voiding is evidenced by the constancy of the creatinine excretion. During this period of decreased urinary volume, a marked diminution in the excretion of total nitrogen occurred. As noted in the protocol, the subject suffered from nausea and general malaise following ingestion of the glutamic acid.

TABLE IX.

*Dicarboxylic Amino-Acids and Amide. Aspartic Acid and Asparagine.*  
200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.
Experiment 17. Aspartic acid.			
	cc.	mg.	mg.
6-7	75	17.2	56.4
7-8	310	21.5	53.0
8-9*	53	22.0	53.5
9-10	37	45.2	56.9
10-11	102	29.7	57.2
11-12	42	17.7	55.3
12-1	52	16.7	50.2
Experiment 21. Asparagine.			
6-7	162	20.2	55.1
7-8	303	22.2	60.6
8-9†	63	25.3	57.8
9-10	85	38.6	56.1
10-11	474	22.5	57.3
11-12	78	18.5	57.7
12-1	152	15.0	54.7

\* 18.4 gm. of aspartic acid (N = 1.9 gm.) at 8.00 a.m. 7 gm. of Na<sub>2</sub>CO<sub>3</sub> were added to effect solution. No nausea. Mild diarrhea lasting several hours.

† 18.4 gm. of asparagine (amino N = 1.9 gm.) taken at 8.00 a.m., dissolved in hot water and ingested while solution was still warm. No abnormal symptoms.

minic acid. It seemed possible that the factor of gastrointestinal irritation might contribute to the increased uric acid elimination. Aspartic acid, a second dicarboxylic amino-acid, and asparagine, its acid amide, however, resulted in increases (Table IX) comparable to those observed in the glutaminic acid experiment. Neither of these substances produced gastrointestinal irritation or ma-

laise, a fact which would seem to rob these of their significance as causative factors in the previous experiments. The diminution of urinary volume was noted in these experiments also. The experiment with asparagine is of interest as showing that masking of one carboxyl group does not alter the power to stimulate uric acid excretion, and that an amide of this type resembles the dicarboxylic amino-acids in its effects rather than the monocarboxylic amino-acids. This is not surprising in view of the ease with which asparagine loses its amide group and is converted to aspartic acid.

Since no digestive processes are required for the utilization of amino-acids it can hardly be considered that the rises in endogenous uric acid observed following the ingestion of four different

TABLE X.

*Experiment 22. Amino-Acids. Sarcosine. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.
	cc.	mg.	mg.
6-7	31	17.4	51.4
7-8	172	24.1	63.6
8-9*	168	20.3	58.8
9-10	210	23.9	61.3
10-11	317	22.2	62.7
11-12	58	17.2	53.7
12-1	30	14.1	54.5

\* 10 gm. of sarcosine taken at 8.00 a.m. N content = 1.6 gm. No ill effects.

amino-acids can be attributed to the work of the digestive glands. The effect is more probably due to a direct stimulation of the body cells by amino-acids or their catabolism products, a stimulation of nuclear metabolism. It is known that amino-acids disappear very rapidly from the blood stream and are stored temporarily in the tissues. If the stimulating influence on nuclear metabolism common to at least four amino-acids is inherently a property of amino-acids as such, substituted amino-acids might be expected to exert a similar influence. If on the other hand, the stimulation is due not to amino-acids as such, but either to the cellular work of their catabolism or to the intermediary products

of their breakdown, a substituted amino-acid which does not follow the normal path of amino-acid catabolism would in all probability be devoid of the power of stimulation. Sarcosine, methyl glycocoll, was selected to test out the effect of a substituted amino-acid (Table X), since it has been observed to pass unchanged through the organism for the most part, although the possibility of its conversion to some extent to creatine has not been entirely excluded. The experiment was not altogether satisfactory, but lack of further material made it impossible to repeat the trial. It is hardly probable that the slight rise the 2nd hour after ingestion was a true rise, the effect of the sarcosine. In any case the effect is too slight to be compared with that of the closely related substance, glycocoll. From this one experiment it would seem that amino-acids as such do not stimulate uric acid metabolism, and that an amino-acid which is not broken down in catabolism is without effect on uric acid excretion.

#### *Ammonium Chloride.*

The first step in the catabolism of the amino-acids within the body is deamination, a reaction which yields as products ammonia and  $\alpha$ -ketonic or hydroxy acids. In order to secure further information as to what reaction or product of reaction is responsible for the effects of amino-acids and proteins on uric acid metabolism, ammonium salts were studied. The ammonia, in amount comparable as to the content of nitrogen with the amino-acids fed, was administered as the chloride, which is less toxic than other ammonium salts. Inorganic ammonium salts are not converted to urea (17, 18), so that this type of ammonium salt should show whether ammonia as such is the factor influencing the changes in purine metabolism observed. No rise in the uric acid excretion above the normal level occurred as a result of the ingestion of ammonium chloride (Table XI). This offers direct evidence that the ammonia from the deamination of the amino-acid plays no rôle in stimulating the output of endogenous uric acid.

The ammonia formed by the processes of deamination is normally converted to urea and excreted as such. It would have been logical at this point to have studied the influence of ammonium carbonate, citrate, or the ammonium salt of some other organic

TABLE XI.

*Experiment 18. Ammonium Chloride. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	mg.	mg.	mg.
6-7	32	20.4	54.2	340.4
7-8	120	22.7	55.8	458.4
8-9*	55	16.5	53.8	395.4
9-10	59	18.2	56.7	454.3
10-11	330	20.1	55.0	607.2
11-12	247	17.0	53.1	553.2
12-1	273	15.5	55.4	543.2

\* 7.4 gm. of  $\text{NH}_4\text{Cl}$  (N = 1.94 gm.) at 8.00 a.m. No toxic symptoms except slight feeling of nausea the first 2 hours.

TABLE XII.

*Urea. 200 Cc. of Water per Hour.*

Time.	Volume.	Uric acid.	Creatinine.
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Experiment 14.

	cc.	mg.	mg.
6-7	59	18.7	61.7
7-8	229	18.3	56.1
8-9*	330	17.1	58.7
9-10	127	20.6	57.5
10-11	177	22.1	60.4
11-12	177	22.1	58.9
12-1	213	13.7	57.5

Experiment 15.

6-7			
7-8	29	15.4	50.9
8-9	192	18.2	55.5
9-10†	96	20.0	56.6
10-11	260	19.5	56.1
11-12	132	16.3	50.9
12-1	209	20.0	57.7

\* 6.6 gm. of urea taken at 8.00 a.m. N content = 3.14 gm. No ill effects.

† 6.6 " " " " " 9.00 " No ill effects.

acid, which is capable of further transformation to urea in the organism. Such an experiment should show whether the conversion of ammonia to urea is a factor related to the problem of the stimulation of uric acid metabolism. Because of the greater toxicity in the required dosage experiments with this type of ammonium salts were not carried out.

### *Urea.*

The ingestion of urea, the end-product of the catabolism of the nitrogenous fraction of the amino-acid molecule, resulted in an excretion of uric acid (Table XII) which did not vary appreciably from the normal. Urea is, therefore, probably not responsible for the rises in uric acid excretion after ingestion of amino-acids and proteins.

### DISCUSSION.

The experiments reported are believed to offer direct evidence against the hypothesis of Mareš that the origin of the increased amounts of endogenous uric acid following the ingestion of protein food is to be attributed *mainly* to the activity of the secretory glands of the gastrointestinal canal. There is no evidence that the presence of amino-acids in the digestive tract stimulates secretion of the juices. On the other hand, data, which indicate that the presence of amino-acids in the system stimulates to a marked degree cellular activity, have been presented by Lusk (14, 15). The increased uric acid excretion which results from the intake of protein food, hitherto explained as a result of stimulation of the secretory glands, can be accounted for equally well as a result of a general stimulation of cellular metabolism by the products of digestion of proteins, the amino-acids. Moreover, the extent of nuclear breakdown necessary to account for the marked increases in the uric acid of the urine reported by many observers would be far too great to be the result of stimulation of so small a proportion of the cells of the body as the cells of the digestive tract. Quantitatively the production of the amounts of uric acid concerned appears possible with a less extensive destruction of the nuclear material of any one set of cells, if the effect is considered the result of a general stimulation of all



cells. It is not necessary to assume that all the endogenous uric acid arises from nuclear breakdown. This as pointed out by Burian (3) would involve too extensive a destruction of nuclear material. A part may originate from the hypoxanthine of muscle tissue. The fact that there is no conclusive evidence that an increased uric acid output follows muscular work is not necessarily a convincing argument against the muscular origin of a part, at least, of the endogenous uric acid. Urinary creatinine is generally considered as a derivative of the creatine of the muscles, yet muscular work does not increase the creatinine content of the urine, nor cause the appearance of creatine. It is believed, however, that the increases in uric acid of the present series of experiments can be considered as originating from nuclear catabolism if the stimulation is considered a general one involving all cells rather than a limited one.

It is also possible as suggested by Taylor and Rose (10) that nuclear anabolism as well as catabolism may be stimulated by the presence of large amounts of amino-acids, in the last analysis, the sources of the nitrogen of the purines of the nucleic acids. A chemical reaction involving the direct synthesis of purines from the four amino-acids ingested in the present series is difficult of conception. Ackroyd and Hopkins (19) have suggested that in the growing rat arginine and histidine may function as purine precursors. The experiments of Lewis and Doisy (20), however, offer no evidence that in man a diet high in protein and high in its content of arginine and histidine increases the uric acid excretion over that eliminated on a high protein diet low in these amino-acids.

It might seem surprising that the difference in time between maximum stimulation of cellular activity, as shown by the changes in the uric acid excretion, by proteins and by amino-acids, is not more marked. The rate of protein metabolism as determined by the excretion of "extra nitrogen" and "extra glucose" in the phlorhizinized dog (21) is nearly as rapid as that of amino-acids. The time required to digest the protein to amino-acids, and to absorb and metabolize these, is only slightly longer than that required for the absorption and metabolism of the amino-acids alone. But slight differences in the time element would accordingly be anticipated between the effects of proteins and amino-

acids on endogenous uric acid metabolism if the influence of the amino-acids is responsible for the changes.

It has been shown in the attempt to find what factor is responsible for the increased uric acid excretion after protein food that the amino-acids are similar in their action to the proteins, and that the ammonia and urea, products of the catabolism of the nitrogenous rest of the amino-acid, are without influence. This would seem to limit the causal agents to two, either the amino-acids or their non-nitrogenous rest,  $\alpha$ -ketonic or hydroxy acids. It was not possible to study the effect of these non-nitrogenous intermediary catabolism products of the amino-acids. Lusk (15), however, concluded that, in the case of glycocoll and alanine, the chemical stimulation of protoplasm, which is responsible for the phenomena of increased heat production (specific dynamic action), results from the action of their intermediary products, glycollic and lactic acids, rather than from the amino-acids themselves. The phenomena of the stimulation of uric acid metabolism by amino-acids run parallel to those of the specific dynamic action of the amino-acids (except in the case of the dicarboxylic amino-acids?) and it is possible that the same chemical factors are responsible for both.

#### SUMMARY.

1. Ingestion of purine-free protein food resulted in an increased uric acid output in the fasting subject, which reached its maximum the 3rd and 4th hours after ingestion of the food. No quantitative differences in the action of three types of protein food, cottage cheese, egg white, and glidine (a wheat protein preparation), in their effect were observed.

2. Amino-acids (glycocoll, alanine, aspartic, and glutaminic acids) also increased uric acid excretion, the maximum effect being produced within 2 hours after ingestion, more rapidly than in the case of the proteins. The stimulation of uric acid metabolism caused by the dicarboxylic amino-acids was more marked than with glycocoll or alanine. Asparagine, the acid amide of aspartic acid, resembled aspartic acid in its action.

3. The effect of the amino-acids is considered to be the result of a stimulation of uric acid production rather than of a more rapid excretion of the uric acid already present in the system,

since successive doses of glycocoll on the same experimental day resulted in an increased elimination of uric acid in each case.

4. Sarcosine, methyl glycocoll, an amino-acid which does not pass through the same path of catabolism as do the other amino-acids, did not influence uric acid excretion.

5. Ammonium chloride and urea, products of deaminization of the amino-acids, were also without effect on endogenous uric acid excretion.

6. Since the secretory activity of the digestive tract is not stimulated by amino-acids, it is believed that the experiments as a whole speak against the hypothesis of Mareš that the secretory activity of the alimentary glands is *mainly* responsible for the increased uric acid excretion observed *after protein ingestion*. It is suggested that the effect is to be considered rather as one due to a general stimulation of all cellular metabolism by amino-acids, the products of the digestion of protein. The relation of the increased uric acid metabolism to specific dynamic action of proteins and amino-acids is discussed.

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# THE ROLE OF INORGANIC SULFATES IN NUTRITION.

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(Received for publication, August 4, 1918.)

The naturally occurring foodstuffs contain inorganic sulfur to such an extent that it is improbable that any normal diet is free from them. There is, however, considerable variation in the amount furnished by given dietaries, and the value of these, in part, may be dependent upon the amount present. A survey of the rations used in studies pertaining to the value of the various foodstuffs in nutrition suggests that such may be the case, if one may judge from the fact that in these more or less sulfate has always been included. Whether or not this inorganic sulfate is really necessary for physiologic processes, or whether it is merely used as a means of carrying the basic ion to which it is attached, is not shown by the experimental data; nor have we been able to find direct evidence that the inorganic sulfates may not be used for building the organic sulfur complexes of the body, although it is generally conceded that these requirements must be met by the organic form.

The importance of the sulfur-containing amino-acid, cystine, has been pointed out by a number of investigators. In studies pertaining to the value of different proteins in nutrition Osborne and Mendel<sup>1</sup> have shown that cystine is a limiting factor in certain cases, for example, casein. The addition of cystine to rations furnishing small amounts of casein produced a resumption of growth in animals previously stunted on similar rations containing no cystine. The addition of small amounts of cystine to diets low in protein, Lewis<sup>2</sup> found diminished the nitrogen elimination in dogs, whereas an equivalent amount of nitrogen in the

<sup>1</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

<sup>2</sup> Lewis, H. B., *J. Biol. Chem.*, 1917, xxxi, 363.

form of tyrosine or glycocoll, when substituted for cystine in similar rations exerted no such influence. Since neither investigation was concerned with the metabolism of sulfates as such, no attempt was made to increase the inorganic sulfate content of the diet as the protein was decreased. In Osborne and Mendel's work the mineral needs of the animals were met by "protein-free" milk which carries small amounts of inorganic sulfates. It is probable that not enough sulfates had been included to make up for the lowered cystine intake on the 9 and 12 per cent casein rations, if such had been possible. Lewis' diets were noticeably low in sulfate on the low protein days.

McCollum and Davis,<sup>3</sup> in an investigation concerned with the influence of minerals on the growth of rats, fed a sulfate-free salt mixture; but since the ration contained approximately 22 per cent protein, furnished by 10 per cent of casein, 10 per cent of milk powder, as well as 64 per cent of wheat, an adequate amount of cystine was supplied. Furthermore, some inorganic sulfates were included in the milk powder.

In the study herein reported we have endeavored to obtain data which might throw light on the part played by the inorganic sulfates in nutrition, and to determine, as far as possible, whether the animal organism is able to synthesize the indispensable cystine from the inorganic sulfates. Young rats weighing between 40 and 80 gm. were divided into groups, two in each case, which were fed rations containing 18, 15, and 12 per cent of casein, respectively. One group in each case received rations which included no sulfates. The other groups were given rations containing sulfur in the form of sulfates equivalent to the amount furnished by a ration consisting of 18 per cent of casein plus a salt mixture similar to that of whole milk. In all other respects the rations were comparable, 1 gm. of each ration supplying 4.86 calories.

The casein used in the rations was obtained from sour milk by acid precipitation. In the process of purification the curd was washed 24 hours in running water, dissolved in 2 per cent ammonium hydroxide solution, reprecipitated with 2 per cent acetic acid, and again washed 48 hours, after which it was dried at about 40°C. and ground to a fine powder.

<sup>3</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, *xxi*, 615.

The food accessories were furnished by butter fat and the alcoholic extract of wheat embryo. The butter fat was obtained by melting butter at 40°C., centrifuging at high speed, and pouring off the supernatant liquid, which was the part used in the experiments.<sup>4</sup> In preparing the water-soluble food accessory the wheat embryo was treated with cold 95 per cent alcohol for 24 hours and then filtered. Distilled water was added to the filtrate, and the alcohol removed by distillation. 9 gm. of wheat embryo furnished the extract for 100 gm. of food.

With the exception of that used with the sulfate-free diets, the mineral mixtures varied with the amount of casein used in

TABLE I.  
*Composition of Salt Mixtures Used in the Rations.*

Salt	Mixture.			
	No. I.	No. II.	No. III.	No. IV.
	gm.	gm.	gm.	gm.
NaCl.....	0.874	0.874	0.874	0.874
KCl.....	0.548	0.548	0.548	0.548
CaHPO <sub>4</sub> ·2H <sub>2</sub> O.....	3.606	3.606	3.606	3.606
Ca lactate.....	0.386	0.386	0.386	0.386
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.438	0	0	0
K <sub>2</sub> SO <sub>4</sub> .....	0	0	0.431	0.562
Mg citrate·14H <sub>2</sub> O.....	0.433	0.848	0.848	0.848
K citrate.....	2.278	2.61	2.107	1.953

the various diets, and were as far as possible comparable to the inorganic content of milk.<sup>5</sup> A few crystals of ferric citrate were added to each ration at the time the food was mixed. The iodine needs of the animals were satisfied by the addition of two drops of a 2 per cent solution of potassium iodide once a week in the distilled drinking water. Table I gives the composition of the salt mixtures used.

Table II gives the composition of the rations.

These materials, together with 1 gm. of filter paper in each case, were mixed with distilled water and heated long enough to

<sup>4</sup> Osborne and Mendel, *J. Biol. Chem.*, 1913-14, xvi, 423.

<sup>5</sup> Forbes, E. B., *Ohio Agric. Exp. Station Bull.* 207, 1909.

gelatinize the starch. The extract of the wheat embryo was added after the mixture had cooled.

The curves of growth of the two groups of animals receiving the 18 per cent casein rations are similar (Chart I, Groups 1 and 2). Those fed the rations containing no sulfates, as well as those receiving the usual amount of sulfates, grew normally and reproduced. Similarly, growth in the two groups receiving the 15 per cent casein ration was comparable. The female in the sulfate-free group gave birth to six young averaging 4.5 gm. each (Chart II, Groups 1 and 2). Apparently inorganic sulfates when added to a diet supplying an adequate amount of a cystine-containing protein fulfills no important nutritive function.

TABLE II.  
*Composition of the Rations per 100 Gm.*

Ration.	Casein.	Lard.	Butter fat.	Cornstarch.	Salt mixture No.	Salt mixture.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		<i>gm.</i>
I	18	15	10	47.44	I	8.56
IV	15	15	10	50.2	III	8.8
V	18	15	10	47.13	II	8.87
VI	15	15	10	50.13	II	8.87
VII	12	15	10	53.13	II	8.87
VIII	12	15	10	53.23	IV	8.77

Especial interest was attached to the animals on the 12 per cent casein ration, for it was believed that here the most conclusive evidence regarding the ability of the animals to synthesize cystine from the inorganic sulfates would be obtained, since it has been shown that normal growth is not produced on rations furnishing only 12 per cent of casein as the sole source of cystine. The curves of growth (Chart III) of our two groups on the 12 per cent rations are strikingly similar, both being considerably below normal. There is no evidence here that rats are able to use the inorganic sulfates to replace the necessary cystine. Had the animals been able to do this in an appreciable degree, there would have been an approach to more nearly normal growth on the part of those animals receiving the liberal amount of the inorganic sulfates.

CHART I

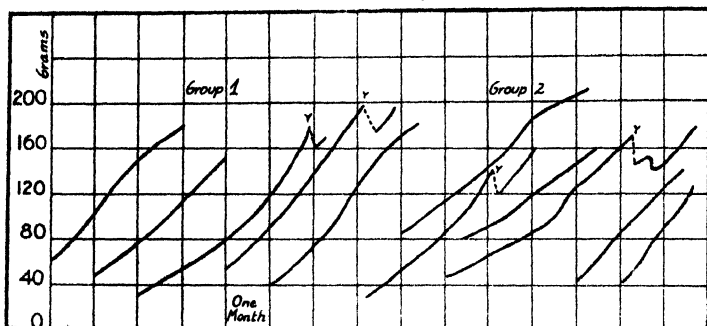


CHART I. Comparable results were obtained in the animals (Group 1) fed rations containing 18 per cent of casein with no sulfate additions (Ration V), and in those (Group 2) receiving an 18 per cent casein ration plus a mineral mixture which included inorganic sulfates (Ration I).

CHART II

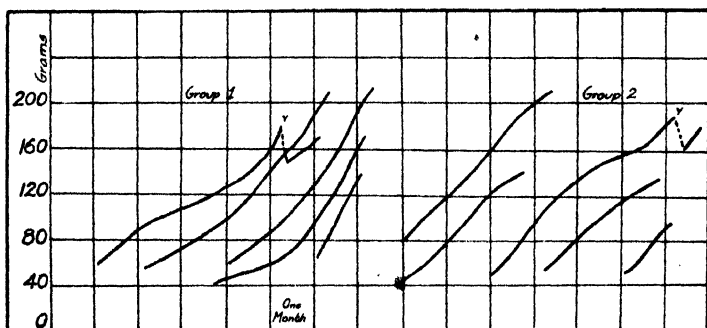


CHART II. A ration containing 15 per cent of casein with no additional sulfur in the form of sulfates supplied enough cystine for growth and reproduction. Growth in Group 2, in which the animals received no inorganic sulfates (Ration VI), was slightly better than in Group 1 where the ration included sulfates (Ration IV). The six young in Group 2 averaged 4.5 gm



CHART III

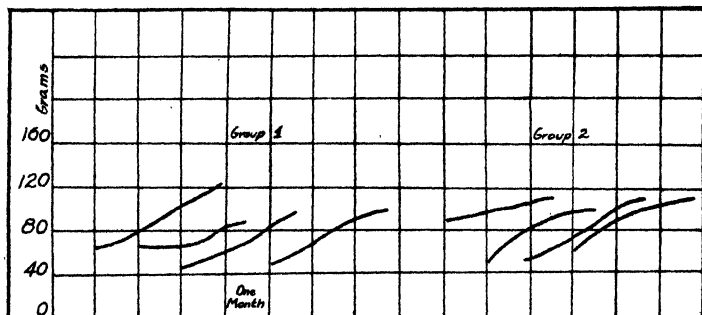


CHART III. Animals (Group 1) receiving rations furnishing 12 per cent of casein together with a mineral mixture supplying no inorganic sulfur (Ration VII) grew quite as well as those rats (Group 2) receiving 12 per cent of casein (Ration VIII) with a mineral mixture containing sulfur in the form of sulfates equivalent to that in a ration made up of 18 per cent of casein plus a salt mixture similar to that of whole milk. The curves of growth in both cases are considerably below normal.

## METHODS FOR THE DETERMINATION OF PHOSPHORIC ACID IN SMALL AMOUNTS OF BLOOD.

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(Received for publication, July 12, 1918.)

As a result of the study of lipoid-phosphoric acid compounds in the blood in various conditions and for other reasons discussed in the succeeding paper, the writer has been led to an investigation of the other forms of phosphoric acid combination occurring in blood, in the hope of making clear the relation of these compounds to fat metabolism and incidentally of getting some information regarding the many other functions of phosphoric acid in the living organism. The methods used in the investigation are reported below. They consist of extensions of a method already used (1) for the determination of lipoid-phosphoric acid compounds ("lecithin") in blood and are based on the nephelometric use of the strychnine molybdate reagent of Pouget and Chouchak (2). This extremely sensitive reagent has been found very satisfactory for use in determining the minute amounts of phosphoric acid in the small amounts of blood which it is expedient to obtain from living animals and especially from human beings. It was first introduced into this country by Greenwald (3) who used it as a colorimetric (really turbidometric) method for the determination of phosphates in blood serum. He used the solution as originally devised by Pouget and Chouchak—a nitric acid solution of the strychnine molybdate. This reagent gives quite good results used either turbidometrically or nephelometrically but has the disadvantage that it must be prepared fresh each time. Kober and Egerer (4) modified it, using hydrochloric acid in place of nitric acid, and adapted the procedure for use in nephelometric determinations. Kober and Egerer's reagent keeps well and is a great improvement on the original.

For use in determining lipid phosphorus ("lecithin") in blood certain modifications of their procedure were, however, found necessary. In the work reported below the reagent is modified with the object of making it stronger and of lessening the manipulations required for producing the precipitation.

The reagent as used was prepared as follows:

*Sodium Molybdate.*—Prepared either according to the direction of Kober and Egerer or, more simply, in the following way. 72 gm. of molybdic acid are mixed with about 300 cc. of water and neutralized with 40 per cent sodium hydroxide (free from all but traces of phosphates). Pure acid requires the theoretical amount of 100 cc., impure samples require less. The molybdate, now in clear solution, is boiled for about a half hour, adding water to keep the volume constant and alkali if the solution becomes turbid. About 1 gm. of talcum powder is added and **after** a further 5 minutes boiling the solution is filtered and the filter washed once with hot water, adding the washings to the main filtrate. After cooling, the solution, containing approximately 100 gm. of sodium molybdate, is ready for use. Most of the molybdic acid now available contains ammonia, which is volatilized during the boiling and allows some of the acid to precipitate, hence the need of more alkali during the boiling.

*Preparation of the Reagent.*—Sufficient of the above solution to contain 30 to 35 gm. of sodium molybdate (or this amount of dry sodium molybdate dissolved in a small amount of water) is measured into a precipitating jar or large beaker (2 liters), and 250 cc. of a mixture of equal parts of concentrated HCl and water are added with stirring. 500 cc. of water are mixed with the solution and 40 to 50 cc. of saturated strychnine sulfate solution slowly added with stirring. 200 cc. more of the dilute acid and 500 cc. more water are added and, after mixing, the turbid solution is allowed to stand over night or longer if convenient. Next day the precipitate has settled and most of the liquid may be poured off clear. The remainder is filtered through a hardened, phosphorus-free filter. For use in the determination, 25 cc. of this solution are taken without further additions.

*Methods.*—The determination of phosphates is based on the precipitation of the phosphoric acid by the reagent as strychnine phosphomolybdate and the measurement of the amount of pre-

precipitate by comparing it nephelometrically with the precipitate produced, under conditions as nearly identical as possible, in a standard phosphate solution. With blood, in most cases a preliminary ashing with sulfuric and nitric acids is necessary to get rid of organic material. Greenwald (3) found that this method of ashing did not always yield accurate results and recommended a modification. In our hands the sulfuric-nitric ashing has been found entirely satisfactory when precautions are taken as mentioned below.

#### *Total Phosphates.*

##### *Whole Blood.*

3 cc. of blood are measured with a pipette into a 25 cc. graduated, glass-stoppered flask, the flask is filled to the mark with water, and the solution well mixed. 1 cc. of the solution (equivalent to 0.12 cc. of blood) is measured into a large (200 × 25 mm.) test-tube, 1.5 cc. of a mixture of concentrated sulfuric and nitric acids and a few glass beads are added, and the whole is heated with a microburner in the hood or by making use of some type of fume absorber (5). The heating is carried out in three stages. In the first stage the mixture is raised to boiling and then the flame turned down until only a slow but constant bubbling takes place. Heating is continued at this rate until red fumes cease to come off. The time required varies with the sample but ordinarily is not more than 15 minutes. In the second stage of heating the flame is increased until the water is driven off and strong heating with volatilization of a part of the sulfuric acid is continued for 8 to 10 minutes, taking care not to heat so strongly that the tube approaches dryness, in which case loss of phosphoric acid may occur. The sulfuric acid solution should now be clear and colorless. If it is brownish in color, a drop of  $\text{HNO}_3$  should be added and the heating continued for 1 minute. In the third stage the mixture is allowed to cool somewhat (for about 2 minutes) and then one or more drops of 1 per cent cane sugar solution is added. (The amount added should be enough to produce a deep browning of the hot solution and the color should disappear when it is boiled. If too much sugar solution has been added and the brown or yellow color persists after a half minute of boiling, a trace of nitric acid should be added

and the boiling continued.) The solution is then boiled until the moisture is gone—about 1 minute—then cooled and about 10 cc. of water are added, rinsing down the sides of the tube. The solution in the tube is neutralized by approximate titration with 10 per cent NaOH (from sodium) using one drop of 0.3 per cent phenolphthalein as indicator, noting the amount of alkali added, then made just acid with a drop or two of dilute sulfuric acid (25 per cent). It is then cooled, transferred quantitatively to a 25 cc. glass-stoppered graduated flask, the tube rinsed several times with water and the washings added to the flask, and the volume made up to the mark with water, and the whole well mixed.

*Standard Phosphate Solution.*—5 cc. of the standard acid potassium phosphate solution (containing 0.15 mg. of  $H_3PO_4$ ) are measured into a 25 cc. glass-stoppered graduated flask, a drop of phenolphthalein is added, and the amount of alkali used in neutralizing the digestion mixture above run in. The solution is then made just acid with the 25 per cent  $H_2SO_4$ , cooled, made up to the mark with water, and well mixed.

*Precipitation.*—25 cc. portions of the strychnine molybdate reagent are measured into each of two 50 cc. glass-stoppered, graduated flasks. 5 cc. of the standard solution are run into one of the flasks, which is kept gently rotating during the addition, and 5 cc. of the test solution similarly added to the other. When the solutions are well mixed they are allowed to stand at least 3 minutes, then filled to the mark, and mixed by inverting several times, after which they are ready to be compared in the nephelometer.

*Reading.*—The nephelometer tubes are filled with the solutions to the same height and to the point at which, when the tubes are in position in the nephelometer, the meniscus is just out of reach of the light. The jacket on the standard tube is set at a convenient point and readings are made as usual.

#### *Plasma.*

0.5 cc. of plasma is measured with a 0.5 cc. Ostwald pipette into a large test-tube, glass beads and 1.5 cc. of the sulfuric-nitric acid mixture are added, and the solution is treated as with whole blood. If a 0.5 cc. pipette is not available, the plasma may be diluted with an equal volume of water and 1 cc. taken.

*Corpuscles.*

Plasma and corpuscles are separated by centrifugation for 10 minutes at about 4,000 R.P.M., the plasma is removed as completely as possible, the corpuscles are washed once by shaking with a volume of 0.9 per cent salt solution equal to the volume of plasma, then centrifuged at once in the same way as before. If this operation is performed quickly, the small remaining amount of plasma is washed out without significant change in the phosphate content of the corpuscles (by dialysis). 0.08 cc. of corpuscles gives a total phosphate content of about the strength of the standard used. To obtain this amount, 1 cc. of the corpuscles is measured with an Ostwald pipette into a 25 cc. glass-stoppered, calibrated flask, the pipette rinsed clean with water, the rinsings are added to the flask, and the mixture is made up to volume with water, and mixed. 2 cc. of this dilution (0.08 cc. of corpuscles) are measured into a large test-tube, then treated as in the case of whole blood.

*Lipoid Phosphoric Acid ("Lecithin").*

The determination of lipid phosphoric acid has been described before (1) but since the method as now used has been modified in some details it is desirable to give it in part here.

*Whole Blood.*—3 cc. of well mixed whole blood is measured into a 100 cc. flask containing about 75 cc. of a mixture of 3 parts alcohol and 1 part ether (both redistilled). The blood is made to enter in a slow stream of drops and the liquid in the flask kept rotating rather rapidly so as to prevent the formation of large aggregates of precipitate which are difficult to extract. The flask and contents are then immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, cooled to room temperature, made up to volume, mixed, and filtered. For the determination 10 cc. (= 0.3 cc. of blood) are measured into one of the large test-tubes, glass beads added, and the whole is evaporated to dryness in a boiling water bath. The tube should be shaken frequently until boiling begins, after which the solution will proceed quietly to dryness. It should be left in the bath a few minutes after it is apparently dry to remove traces of alcohol which would interfere

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with the subsequent oxidation. 1.5 cc. of the sulfuric-nitric acid mixture are added, distributed by shaking to the material on the sides of the tube, and the mixture is digested in the same way as directed for total phosphates.

*Plasma.*—2 cc. of plasma are measured into 35 to 40 cc. of alcohol-ether in a 50 cc. flask as described above and the whole process is carried out as for whole blood. For the determination 15 cc. is required (0.6 cc. of plasma).

*Corpuscles.*—Corpuscles are hemolyzed by dilution with an equal volume of warm water and allowing to stand for 10 minutes, then 3 cc. of the dilution are measured into 35 to 40 cc. of alcohol-ether in a 50 cc. flask and treated as with whole blood. There is a tendency for the corpuscular precipitate to mass together when it settles out and the flask should be well shaken occasionally during a half hour after which the extraction may be proceeded with as before. For the determination 10 cc. of the extract (0.3 cc. of corpuscles) are used.

### *Acid-Soluble Phosphoric Acid—Inorganic and Other Forms.*

#### *Whole Blood.*

The blood is first laked by the addition of an exactly equal volume of warm (40°C.) water and letting stand for about 10 minutes with occasional shaking. Of this mixture 5 cc. are measured with a pipette into 15 cc. of acid ammonium sulfate (see acid ammonium sulfate in the notes below, page 41) in a 25 cc. glass-stoppered graduated flask. The blood is added slowly and the liquid in the flask kept rotating during the addition, after which the flask is filled to the mark with the ammonium sulfate, well mixed and let stand with occasional shaking for at least 10 minutes. The liquid is then filtered through a phosphate-free filter. The filtrate is clear, colorless, and free from protein. In this filtrate determinations are made of acid-soluble, inorganic, and, by difference, other forms of phosphoric acid.

*Inorganic Phosphoric Acid.*—10 cc. of the filtrate (= 1 cc. of original blood) are measured into a 25 cc. glass-stoppered flask, made to the mark with water, and mixed. The standard is prepared by measuring 3 cc. of the standard phosphate solution (0.09

mg. of  $\text{H}_3\text{PO}_4$ ) into a similar flask, adding 8 cc. of the acid ammonium sulfate (to balance the salt content of the test solution), filling to the mark with water, and mixing. Determinations are made with 5 cc. of this dilution as in total phosphates.

*Acid-Soluble Phosphoric Acid.*—2 cc. of the filtrate (0.2 cc. of blood) are measured into one of the large test-tubes, glass beads and 1.5 cc. of the sulfuric-nitric acid mixture added, and the whole is digested with heat in the same way as for total phosphates, **except that since the amount of organic material is very small, the first stage of heating may be passed over quickly.** The neutralization and subsequent treatment are the same as for total phosphates. For a standard solution measure 3 cc. of the standard phosphate solution (0.09 cc. of  $\text{H}_3\text{PO}_4$ ) into a 25 cc. glass-stoppered flask, add about 1.5 cc. of the acid ammonium sulfate and the amount of alkali used in neutralizing the test solution. Neutralize with acid as usual, then make to the mark and mix. Subsequent treatment is as with total phosphates.

*Other Forms of Phosphoric Acid.*—Obtained by subtracting inorganic from acid-soluble phosphoric acid.

### *Plasma.*

3 cc. of plasma are run slowly into 20 cc. of the acid ammonium sulfate solution in a 25 cc. glass-stoppered graduated flask, the volume is made to the mark with water, the whole mixed, and let stand with occasional shaking for at least 10 minutes. It is then filtered. After the filter has drained, it should be folded in the funnel and pressed out with a clean stirring rod to get as much filtrate as possible. The filtrate is clear and colorless and contains no detectable protein. With this filtrate determinations are made of inorganic and acid-soluble and other forms as before.

*Inorganic.*—(a) 10 cc. of the filtrate (= 1.2 cc. of plasma) are measured into one of the 25 cc. flasks and made to the mark with water. A standard is prepared by adding to another flask 3 cc. of the standard phosphate (0.09 mg. of  $\text{H}_3\text{PO}_4$ ) and acid ammonium sulfate equal to that present in the test solution (8 cc. is a sufficiently close approximation). The flask is filled to the mark with water, and precipitations and readings are made



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as usual. The determination should be made promptly after filtering. (b) Where many determinations are to be made it is advisable to make a special standard,—when determinations may be made more simply as follows:

*Standard.*—Made by measuring 2 cc. of the strong stock standard phosphate solution (= 1.2 mg. of  $\text{H}_3\text{PO}_4$ ) into a 100 cc. flask, adding 80 cc. of the acid ammonium sulfate, and making up to 100 cc. with water. Of this standard 5 cc. contains 0.06 mg. of  $\text{H}_3\text{PO}_4$ . For the determination 5 cc. of the filtrate (= 0.6 cc. of plasma) are measured directly into 25 cc. of the strychnine molybdate reagent in one 50 cc. graduated flask, 5 cc. of the standard solution into another, and after mixing and standing 3 minutes the flasks are filled to the mark, the solutions mixed, and determinations made.

*Acid-Soluble.*—10 cc. of the filtrate are measured into one of the large test-tubes, glass beads and 1.5 cc. of sulfuric-nitric acid mixture are added, and the mixture is digested. The digestion presents some difficulties because of the large amount of ammonium sulfate present. The first stage is passed over quickly, then in the second stage of heating when the mixture thickens and begins to foam, the heat is moderated and so continued until foaming ceases and the salt fuses to a small volume in the tube. Heating is then carried on at a rate just sufficient to prevent loss of ammonium sulfate from the tube by volatilization for about 10 minutes. The tubes are then cooled, treated with one drop of 0.3 per cent cane sugar solution, and reheated in the regular way. After dissolving in water the solution is titrated with alkali, noting the amount used. The standard contains 3 cc. of the standard phosphate solution, 8 cc. of the acid ammonium sulfate, and the amount of alkali used to neutralize the test solution. The mixture is neutralized and the phosphoric acid determinations are carried out in the regular way.

### *Corpuscles.*

5 cc. of corpuscles are measured with a pipette into a 10 cc. glass-stoppered graduated flask and the pipette is rinsed clean with small portions of warm water. The rinsings are added to the flask, the whole is made to volume, mixed, and let stand

with occasional shaking for at least 10 minutes to allow laking of the corpuscles. 5 cc. of this dilution are run slowly, with shaking, into 15 to 18 cc. of the acid ammonium sulfate in a 25 cc. flask, the volume is made to 25 cc. with the ammonium sulfate solution, and the whole well mixed. Of this mixture 10 cc. are measured out and used for the determination of acid-soluble phosphoric acid as directed below. The remainder is allowed to stand at least 10 minutes, then filtered, and determinations are made of inorganic phosphates.

*Inorganic.*—5 cc. of the filtrate (= 0.5 cc. of corpuscles) are used for the determination which is carried out as in method (b) for plasma, using the same standard. Determinations should be made at once after filtering since in certain cases and particularly in warm weather the values have been found to increase on standing, probably at the expense of the unknown acid-soluble phosphoric acid compound.

*Acid-Soluble.*—10 cc. of the ammonium sulfate-corpuscle mixture are measured into a small flask and immersed for 2 minutes in boiling water. After cooling it is poured into a 25 cc. graduated flask, the small flask is rinsed with 10 cc. of the acid ammonium sulfate, and the rinsings are added to the main portion in the 25 cc. flask. The volume is made up to the mark with water, mixed, and filtered. For the determination 2 cc. of the filtrate (0.08 cc. of corpuscles) are measured into one of the large test-tubes, glass beads and 1.5 cc. of the sulfuric-nitric acid mixture are added, the whole is digested, and determinations are made as with total phosphates, the small amount of ammonium sulfate present not interfering. For the standard, 5 cc. of the standard phosphate (0.15 mg. of  $H_3PO_4$ ) are used and to it are added 1.5 cc. of the ammonium sulfate solution; then it is treated with alkali, neutralized, etc., as in the regular determinations.

#### *Notes on the Methods.*

*Solutions Required for These Determinations.*—1. The strychnine molybdate reagent as described.

2. Acid ammonium sulfate. Saturated ammonium sulfate, free from all but traces of phosphate, to which have been added 15 cc. of glacial acetic acid per liter.

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3. Standard phosphate. (a) Stock standard, containing in 100 cc. 0.0834 gm. of pure acid potassium phosphate. (b) Standard for use: dilute 25 cc. of the above to 500 cc. Each 5 cc. of this standard contains 0.15 mg. of  $H_3PO_4$ . The dilute standard has been found to deteriorate in hot weather and should be made up at least once a month.

4. Sodium hydroxide (from sodium) 10 to 20 per cent.

5. Concentrated sulfuric and nitric acids free from all but traces of phosphates (see below).

6. Dilute sulfuric acid. 1 part of the concentrated acid and 3 parts water.

Blanks run with the acids, alkali, and ammonium sulfate should show only a slight cloudiness on standing for 1 hour with the reagent. The ordinary highest grade reagents obtainable up to the present time have been found satisfactory with the exception of the sodium hydroxide which should be made from the metal. If the distilled water is stored in metal tanks it may contain lead which is itself a fairly good precipitant for phosphates. The water should therefore be stored in glass.

Because of the minute amounts of phosphoric acid determined the greatest care should be used to avoid contamination, and free use should be made of good distilled water. For the same reason glass-stoppered measuring flasks should be used throughout.

*Reagents.*—With impure samples of molybdic acid a highly colored solution is often obtained which is difficult to use. It has been found that if the colored solution is exposed to sunlight for a time the color fades and, if the reagent is then taken out of the direct light, does not reappear. (If placed in the dark, the color reappears to some extent, and, if exposed to sunlight too long, the reddish purple color changes to a deep yellow which is almost as difficult to work with as the original color.) If pure molybdic acid is used, as recommended by Kober and Egerer, the reagent is colorless but with the above precautions it has been found possible to use acid of varying degrees of purity.

*Methods.*—The quantities of blood taken are based on the average values for normal human blood and variations in amount of blood or strength of standard may be necessary in special cases.

The tubes with the beads should be given a preliminary "steaming out" by boiling concentrated sulfuric acid in them for at least half an hour. Four beads about 3 mm. in diameter have been found the most satisfactory number for each tube in the determinations. With use they become etched by the strong acid and are then more effective in promoting even boiling than when new.

If the heating of the blood with the acid mixture is hastened too much, the nitric acid may be driven off before oxidation is complete—as shown by a darkening of the solution. If this occurs one or two drops of nitric acid are added, and the heating is continued.

The use of the cane sugar is necessary because of the formation of a varying amount of some compound of phosphoric acid (probably a nitric acid compound) which does not precipitate with the reagent and which is destroyed by organic material added. In the determination of lipoid phosphoric acid the sugar appears to be less necessary, but in determinations of total phosphates low values are invariably obtained unless it is used, and it is safest therefore to use it as a routine procedure in all determinations. The formation of this compound may be the reason why Greenwald found the sulfuric-nitric acid digestion unsatisfactory.

During the boiling of a  $\text{H}_2\text{SO}_4$ -water mixture, first the excess of water is boiled off, then the water vapor is succeeded by a dense white cloud of mixed water and sulfuric acid vapor, and finally by the much thinner vapor of the sulfuric acid alone. In this final stage a clear zone appears between the liquid and the vapors above. The appearance of the clear zone indicates that the heat is strong enough.

Accurate nephelometric measurements are possible only when the size of the particles of the suspension is approximately the same in both standard and test solutions. This similarity is secured by adjustments of conditions as regards salt content, temperature, etc., which are made as nearly as possible the same in both solutions.

3 minutes is the minimum time for the precipitation to come to the point where it may be measured. The length of time that the solutions may stand and still give accurate readings has not been

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determined, but in solutions no more than 25 per cent apart no significant change has been found in 20 minutes.

The jackets of the nephelometer should fit snugly at the top so that the light is cut off sharply at that point and there should be the minimum of play in either tubes or jackets, otherwise consistent readings are not obtained. It is rarely possible to get tubes or fittings sufficiently alike so that when both tubes are filled with the same solution and the standard set at a given point, say 25 mm., the test solution will read 25 mm. Kober's suggestion has always been followed in this connection; *i.e.*, set the test solution at the required point (say 25 mm.) and adjust the standard until the two fields are the same. Then set the standard at that point and call it 25 mm. The correctness of the position should be tested frequently.

The Richard's type of nephelometer (with moving jackets), made by adapting a Duboscq colorimeter (6), was used throughout the work. This type of nephelometer is believed to be preferable to the plunger type because no foreign body with its possible sources of contamination comes in contact with the solutions. The danger will be realized when attention is directed to the fact that in ordinary determinations the amounts measured are about 0.03 mg. of  $\text{H}_3\text{PO}_4$  in 50 cc. of solution.

*Corrections.*—For reasons which have been discussed in part elsewhere (1, 4) the readings obtained in the nephelometer are not exactly proportional to the amounts of phosphoric acid in the solutions. Strong solutions appear stronger and weak solutions weaker than they should. Different schemes for correction of the readings have been suggested, none of which has been found entirely satisfactory and for exact work the necessity for correction is avoided by adjusting the strength of the standard until the correction falls within the limit of error of the method. Solutions up to about 25 per cent stronger or weaker than the standard may be compared without correction. Solutions from 25 to 50 per cent above or below the standard may be read with correction with an accuracy of 3 to 5 per cent of the theoretical value. It is not thought advisable to attempt comparison of solutions varying more than 50 per cent from the standard. The standard phosphate solution contains 0.03 mg. of  $\text{H}_3\text{PO}_4$  per cc. and if the regular 5 cc. make a standard more than 50 per cent

above or below the test solutions, a fresh standard should be made with 2 to 10 cc. of the standard, which will give solutions falling within the ordinary range of blood phosphates. Adjustment of the salt content must of course be made as with the ordinary standard.

In the determination of lipid-phosphoric acid it makes no difference whether the second part of the extraction—the heating—is done at once or after some time. Ordinarily it has been found convenient to keep the flasks in a cool, dark place for a week or more until a suitable number have accumulated before finishing the process. The extracts kept cool in the dark and tightly stoppered will remain unchanged for 2 or 3 months. They may also be used for determination of other blood lipoids as well as “lecithin.”

Alcohol-ether has been found to make an exact separation of the lipid from the acid-soluble forms of phosphoric acid combination in blood. Tests have been made for water-soluble phosphoric acid in these extracts as described (1) and also in the following way. 100 cc. of the alcohol-ether extract (of blood, plasma, or corpuscles) were evaporated to dryness on a water bath. 25 cc. of water were added, and the solution was raised to boiling with stirring. The extract was saturated with ammonium sulfate (to salt out the lecithin, etc.), filtered, and the filtrate was tested for inorganic and organic phosphoric acid. This test has been made a number of times and at no time was there found more than a trace of phosphoric acid—which may have originated from the lecithin—even in cases of severe nephritis where the inorganic phosphate was known to be much above the normal value. As further confirmation of the completeness of the separation, the sum of acid-soluble and lipid phosphoric acid has been found in both plasma and corpuscles to be very nearly equal (probably within the limits of error of the various determinations) to the total phosphates. Hence if one is known the other may be determined by difference with a good degree of accuracy.

Because of the tendency of the unknown phosphoric acid constituent of the acid ammonium sulfate extract (especially that from the corpuscles) to decompose, yielding phosphoric acid, the

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determinations of inorganic phosphate should be carried out at once after filtering.

Cold (room-temperature) extraction with acid ammonium sulfate for at least 10 minutes was found to give complete extraction of both inorganic and unknown acid-soluble phosphoric acid in whole blood and plasma but not of the unknown in the corpuscles. For this reason heating for 2 minutes was necessary to recover all of this substance from the corpuscles. Whether heating increased the amount of inorganic phosphoric acid extracted from corpuscles could not be determined because heating decomposes the unknown, yielding phosphoric acid. For that reason inorganic phosphates could be determined only in the extract made in the cold. Since, however, cold treatment gave complete extraction of inorganic from plasma and since added phosphate was completely recovered from corpuscles by the cold extraction, it is believed that it is adequate for the determination of inorganic phosphates in the corpuscles.

To avoid a multiplicity of standards, directions are given for the use in most cases of a single standard. In case a single determination is to be made on a large number of samples it is better to make a special standard as in method (b) for inorganic phosphates in plasma.

### RESULTS.

In reporting results of the use of these methods with blood the values are given in terms of phosphoric acid, since this is the only form of phosphorus known to occur in living beings. The values divided by 3.2 give a good approximation to the values expressed as phosphorus and the values for lipid phosphoric acid multiplied by 8 to those for "lecithin."

The checking of the nephelometric method for determining phosphoric acid against other methods has already been reported (1, 4) as has also the determination of lipid phosphoric acid (1). The following results will indicate the possibilities of the remaining methods.

*Recovery of Added Phosphates.*

	Added phosphate.	Present in blood.		Blood and added phosphates.		
Total phosphates.						
	mg.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Beef blood.....	15	62.7	62.9	77.7	78.1	
“ plasma.....	20	50	49.5	70.2	69.4	
Acid-soluble phosphates.						
Beef blood.....	14.1	32.8	32.8	46.6	47.4	
“ plasma.....	14.1	24.5	24.8	39.0	38.7	
“ corpuscles (laked).....	28.2	39.0	39.6	68.0	67.2	68.4
Inorganic phosphates.						
Beef blood.....	14.3	20.0		34.0		
“ plasma.....	13.7	20.0	20.7	33.3	33.6	
“ corpuscles (laked).....	28.0	10.1		38.6		

*Calculated and Determined Values on Whole Blood, Plasma, and Corpuscles.*

Knowing the percentage of corpuscles, and the phosphate values for whole blood and plasma, the values for corpuscles may be calculated, and the comparison of the found and calculated values is a good check on the accuracy of the determinations. They should check within 5 per cent. The following are results for total phosphates in mg. per 100 cc.

	Corpuscles.	Plasma.	Whole blood.	Corpuscles.	
				Calculated.	Determined.
	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Sample I (human).....	43.6	62.0	130	218	210
" II ".....	34.4	71.0	130	243	250
" III ".....	48.0	42.5	120	204	205
" IV ".....	46.5	43.5	127	223	221



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## THE DISTRIBUTION OF PHOSPHORIC ACID IN NORMAL HUMAN BLOOD.

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(Received for publication, July 13, 1918.)

In the last few years considerable evidence has accumulated to show that the lipid compounds of phosphoric acid of the type of lecithin are probably of great importance in the metabolism of the fats. For example a marked increase has been observed in lecithin in the blood and particularly in the corpuscles during fat absorption (1). In those pathological conditions where the blood lipoids are abnormal, as in diabetes (2) and anemia (3) the values for lipid phosphorus follow in general those for total lipoids. The increase in fat which takes place in the blood of hens at the time of egg production has been found to be accompanied by increased lipid phosphorus (4). Then there is the very significant observation of Meigs and Blatherwick (5) that during the secretion of milk in cows the blood leaving the milk gland contained less lipid phosphorus and more inorganic phosphorus than the blood entering the gland. Also that the decrease in lipid phosphorus was sufficient to account for the fat of the milk—clearly indicating that the milk fat originated in the lipid-phosphorus compounds of the blood. On the other hand, in very severe diabetic lipemia (6), the increase of lipid-phosphoric acid compounds<sup>1</sup> was relatively less than that of some of the other lipid constituents; *e.g.*, cholesterol. The question arose whether the reason for the relatively low value may have been the lack of phosphoric acid available for lipid combination. This involved the general problem of the relation of the lipid phosphoric acid to other forms of organic phosphoric acid in the blood. These and

<sup>1</sup> In the report of this result (124 Cl. 786) the values given for lecithin in whole blood, plasma, and corpuscles were, through a miscalculation, just half the true values.

other considerations, as for example the part which phosphates take in the regulation of the reaction of the blood and tissues, combined to render the study of the distribution of phosphoric acid in the blood a very desirable form of investigation.

The older work on this subject consisting mainly of determinations of total phosphates in blood and blood plasma or serum has been recently reviewed by Forbes and Keith (7). A summary of the work on lipoid-phosphorus compounds has been given in an earlier paper (8). Recent data regarding the distribution of phosphorus compounds in blood serum have been supplied by Greenwald (9), Marriott and coworkers (10, 11), and by Feigl (12). Greenwald found that the phosphorus compounds of blood and blood serum could be divided into three classes—lipoid, acid-soluble, and protein. The last was present only in small quantities in blood and was in negligible amounts or absent in blood serum. The lipoid phosphorus was completely separated with the protein by the acid precipitation. Greenwald's values for acid-soluble phosphorus varied between 2 and 4.5 mg. per 100 cc. (= 6.3 to 14 mg. of  $\text{H}_3\text{PO}_4$ ) and the lipoid phosphorus between 6.6 and 10.7 mg. per 100 cc. (= 21 to 34 mg. of  $\text{H}_3\text{PO}_4$ ) in four normal persons presumably fasting, although no information is given on this point. Total phosphorus in these persons varied between 9.5 and 13.6 mg. per 100 cc. (= 30 to 44 mg. of  $\text{H}_3\text{PO}_4$ ), and the sum of lipoid and acid-soluble was about equal to the value for total phosphorus. Greenwald's method for determining acid-soluble phosphorus was by precipitation of the protein with dilute acetic and picric acids, filtering, and then ashing the filtrate. The values obtained by this procedure would be very close to those given as acid-soluble in the work reported below. They include not only inorganic but probably also another form of phosphoric acid combination (see below).

Feigl found that the normal "soluble" phosphorus is less than 4 mg. per 100 cc. of plasma (= about 13 mg. of  $\text{H}_3\text{PO}_4$ ) in 90 per cent of his normals but may occasionally go much higher. In a later communication he draws a distinction between *o*-phosphate and acid-soluble phosphates in which the acid-soluble is greater by about 15 per cent. The original papers are not available.

Marriott and Howland and Marriott and Haessler found 1 to 3.5 mg. of inorganic phosphorus (= 3.2 to 12 mg. of  $\text{H}_3\text{PO}_4$ ) per 100

cc. of human blood serum. Much larger amounts were found in nephritics with acidosis.

The methods used for the present work have been described in the preceding paper and were adapted for determination of the distribution of phosphoric acid in a single sample of blood as follows:

15 cc. of freshly drawn citrated blood were separated into corpuscles and plasma by centrifugation for 10 minutes at about 4,000 R.P.M., the plasma was removed as completely as possible, and the corpuscles were washed once by rapidly mixing with normal saline and at once centrifugating as before. After removal of the wash liquid, 5 cc. of the corpuscles were measured with a pipette into a 10 cc. graduated flask, the pipette was rinsed clean with small portions of water, and the washings were added to the flask which was then filled to the mark and well shaken. After standing for at least 10 minutes with occasional shaking, 5 cc. of the mixture was used for acid-soluble and inorganic phosphates, 3 cc. in 50 cc. of alcohol-ether for lipid phosphoric acid and 1 cc. in 25 cc. of water, of which 4 cc. were taken, for total phosphates. Of the plasma, 3 cc. were used for acid-soluble and inorganic, 2 cc. (in 50 cc. of alcohol-ether) for lipid, and 0.5 cc. was used for total phosphates.

Since changes in the distribution of the phosphoric acid have been found to take place in the blood and particularly in the corpuscles on standing, determinations, especially of inorganic phosphates, were carried out as soon as possible after drawing the blood. Some of the samples obtained from a distance were put on ice at once and kept cold until used.

Because of the probable great influence of food on the phosphate content of the blood, and also in order to preserve the continuity of the series begun with the blood lipoids, as well as to obtain a normal base for study of changes produced by various factors, blood samples were always taken in the postabsorptive condition — before breakfast.

The normal men were students in medicine at this School, the women were in part surgical cases as noted, from the wards of the Massachusetts General Hospital,<sup>2</sup> with presumably normal metabolism, and the others were normal healthy women.

The results of the analyses expressed in mg. of phosphoric acid per 100 cc. of plasma or corpuscles are given in Table I.

<sup>2</sup> To all of these and to Dr. W. Denis of this School, who obtained most of the women's samples for me, I acknowledge my indebtedness and express my thanks for their cooperation.

## DISCUSSION.

The values in general are within the limits reported by other investigators, with the exception of those for lipid phosphoric acid—some of which are also different from our own previously published normal values (8). In the plasma, for the men the average is 22 mg. of  $H_3PO_4$  per 100 cc., as compared with the previously found average of 26 mg. and Greenwald's average of 25 mg. The relatively large number of low values found in this series explains the lowered average. One value (No. 715) is considerably higher than those previously found and since this value seems exceptional it is not included in the average. For women the average is very nearly that previously reported—24.9 mg. of  $H_3PO_4$  per 100 cc., as compared with the previous value of 23.8 mg.

In the corpuscles, the lipid phosphoric acid values for the men average 57 mg., with variations of 44 to 67 mg., as compared with the previous average of 50 with variations of from 44 to 55 mg. of  $H_3PO_4$  per 100 cc. For the women the average is 57 with variations of 47 to 63 mg., which is practically the same as previously reported—average 55 and variations of from 49 to 60 mg. per 100 cc. It should be noted that the values given for lipid phosphoric acid in this paper are the result of direct determination; those in the earlier work, of calculation from values for whole blood and plasma.

Variations in all values are in general more marked in the plasma than in the corpuscles. In this connection the practically identical average values for all constituents in the corpuscles of men and women is probably more than a coincidence and both observations are in agreement with the idea that the corpuscles are like other cells in having more or less constant values for their various constituents, while the plasma, being the medium of exchange between the exterior and the blood and tissue cells, is naturally more variable in composition.

The constituent showing the greatest percentage variation in the plasma is the so called "Other forms"—unknown phosphoric acid combinations. Next in variability is the lipid phosphoric acid which is the greatest factor in producing the variations in total phosphates. In the corpuscles the most variable constituent is the inorganic phosphoric acid.

**TABLE I**  
*Phosphoric Acid Compounds in Normal Human Blood. Mg. of  $H_2PO_4$  per 100 Cc.*

	Plasma.					Corpuscles.				
	Total.	Acid-solu-ble.	Inorganic.	Lipoid.	Other forms.	Total	Acid-solu-ble.	Inorganic.	Lipoid.	Other forms.
<b>Men.</b>										
BFY 600.....	43.5	9.3		33.3		221	160		55.2	
DJ 702.....	28.2	10.0	8.2	20.0	1.8	195	151	20.3	48.6	130.7
DV 703.....	27.3	10.8	9.0	17.2	1.8	200	150	13.3	46.5	136.7
FJT 705.....	29.3	10.7	6.7	19.0	4.0	185	140	16.2	46.5	123.8
BFS 706.....	29.6	10.3	9.7	21.2	0.6	250	192	20.7	57.8	171.3
PWS 707.....	28.3	11.6	10.1	18.5	1.5	210	160	22.6	43.5	137.4
CY 708.....	30.0	11.4	8.3	17.5	3.1	210	152	13.7	44.2	138.3
ACB 709.....	24.3	9.0	7.5	16.0	1.5	225	156		61.7	
ALA 710.....	34.0	13.7	12.0	21.3	1.7	260	200		60.6	
AT 711.....	29.3	8.0	7.6	20.5	0.4	280	214	12.2	66.6	201.8
RMS 712.....	40.0	7.5	6.0	33.3	1.5	270	210	25.0	62.5	185.0
RJP 713.....	33.3	10.0	8.0	23.5	2.0	275	208	17.0	66.6	180.7
CHH 714.....	29.3	8.7	8.5	22.8	0.2	258	197	15.4	65.5	181.6
FLI 716.....	35.7	12.5	11.5	21.7	1.0	292	234	27.3	62.5	206.7
PW 720.....	33.5	10.0	9.1	26.7	0.9	238	184	20.0	50.0	164.0
LD 717.....	35.3	10.7	7.0	21.7	3.7	315	250	12.0	63.5	238.0
EHL 715.....	54.2*	12.5	10.7	40.0*	1.8	325	250	26.1	66.6	223.9
<b>Women.</b>										
CHB 718.....	33.5	9.4	8.0	23.5	1.4	260	205	16.7	47.0	188.3
NB 719.....	31.0	12.0	11.0	19.0	1.0	265	202	18.8	54.5	183.2
S 724.....	33.0	12.2	11.5	24.3	0.7	247	182	18.2	58.9	163.8
M 725.....	41.0	13.8	12.6	27.2	1.2	250	179	17.2	62.5	161.8
G† 721.....	33.3	11.2	9.7	21.5	1.5	259	200	11.8	55.5	188.2
H† 722.....	36.8	10.0	8.5	25.0	1.5	262	206		62.5	
P† 723.....	39.3	14.0	10.0	29.0	4.0	259	196	9.8	54.8	186.2
R† 726.....	37.5	13.8	13.8	25.0	0.0	250	183	18.1	59.5	164.9
D† 727.....	34.3	14.3	13.7	23.0	0.6	218	160	26.3	59.8	133.7
K† 728.....	37.5	14.0	13.3	22.2	0.7	218	159	14.7	51.3	134.3
<b>Averages and variations.</b>										
<b>Men.</b>										
Low.....	24.3	7.5	6.0	16.0	0.4	185	140	12.0	43.5	123.0
Average.....	32.0	10.4	8.7	22.1	1.72	248	188	18.7	57.0	172.0
High.....	43.5	13.7	12.0	23.3	4.0	325	250	27.3	66.6	238.0
<b>Women.</b>										
Low.....	31.0	9.4	8.0	19.0	0.0	218	160	9.8	47.0	133.7
Average.....	36.2	12.4	11.2	24.9	1.26	249	187	15.7	56.6	167.0
High.....	41.0	14.3	13.8	29.0	4.0	265	206	26.3	62.5	188.3

\* Not included in the average.

† Surgical patients.

The sum of acid-soluble and lipid phosphoric acid has in almost all cases been found to be equal to the value for total phosphates, within the limits of experimental error, so that the presence of any considerable quantity of other forms of phosphoric acid combination not included in these groups,—such as Greenwald's protein-phosphorus compounds or nucleoprotein,—seems doubtful.

There is a relatively great excess of all the groups of compounds in the corpuscles as compared with the plasma: total phosphates five to seven times, "unknown" fifty to one hundred times, lipid two to three times, and inorganic twice as much. For this reason and because of the relative constancy of values in the corpuscles, any active interchange of these compounds between corpuscles and plasma seems open to question. The only known instance of an interchange of phosphoric acid compounds is the case of "lecithin" which accumulates in the corpuscles during fat absorption and disappears from them in the course of a few hours (1). It is possible that the compounds in the corpuscles are different from those in the plasma or that the corpuscles may have the power of selective retention of these substances such as they are known to have in the case of potassium. On the other hand the life of a corpuscle is known to be short and it is not unlikely that when it is broken up the relatively large amount of the various phosphoric acid compounds which it contains is liberated into the plasma and eventually into the urine, thus accounting for part at least of the organic phosphorus of these fluids.

Perhaps the most interesting fact brought out by these analyses is the presence in the plasma to a slight extent (up to 10 per cent) and in the corpuscles to a very marked degree (60 to 80 per cent of the total phosphoric acid) of the unknown compound (or compounds) of phosphoric acid designated in the table as "Other forms". This compound, according to our present information, is soluble in dilute acids (acid ammonium sulfate, acetic, and nitric acids) but insoluble in alcohol-ether. A few observations indicate that it is an unstable substance, decomposing in the blood on standing and by dilute acids after extraction yielding phosphoric acid. It is also slowly dialyzable. Nothing is known regarding its chemical nature; in fact its presence in quantity has

not been suspected. Glycerophosphoric acid has been reported (13) in the blood in minute amounts and Greenwald (9) has suggested that some at least of his acid-soluble phosphorus in serum may be glycerophosphoric acid. He suggests also inosinic acid, although, as he has pointed out, the serum of the animals with which he worked does not contain purine nitrogen corresponding to the amount of phosphoric acid (an objection which would not hold as regards the relatively small amount of the unknown compound present). An attractive possibility is that the unknown substance in the corpuscles is indeed inosinic acid or some similar residue of the nucleoprotein of the nucleus which is no longer present in the mammalian erythrocytes. However, a few observations on chicken erythrocytes (nucleated) indicate that this acid-soluble phosphoric acid compound is present in abundance in addition to the nucleic acid phosphorus. The large amount of organic phosphorus in the corpuscles is also very significant in view of the fact that the corpuscles have been found to be one of the places of formation of "lecithin" during fat absorption and it seems likely that this substance is the source of at least the phosphoric acid of the lipid-phosphorus compounds.

The presence of inorganic phosphates in the blood has been disputed. Taylor and Miller (14) found only negligible traces, thus confirming the observation of Gürber (15). Greenwald (9) found considerable acid-soluble phosphate in blood serum, which he believed to be mainly inorganic. Bloor (16) found that no water-soluble phosphorus was extracted from blood by alcohol-ether although inorganic phosphates are measurably soluble in that solvent. The results reported above appear to furnish satisfactory evidence of the presence of inorganic phosphates in both plasma and corpuscles, since the extract with cold, weakly acid ammonium sulfate gives a precipitate with the reagent, which only free phosphate is known to do. The only alternative is that the extracting solution decomposes some form of phosphoric acid combination liberating phosphoric acid—a supposition which seems unlikely because of the mildness of the solution and the short time it is in contact with the blood.

A possible explanation of the varying findings with regard to these substances is that there is normally present in blood suffi-



cient calcium and magnesium to combine with all the phosphoric acid present and that at the slightly alkaline reaction of the blood practically all the inorganic phosphate would be combined with these substances in the dibasic form, which is relatively insoluble. In this case the treatment with dilute acid would bring it into solution.

The relatively small amount of inorganic phosphate in plasma and corpuscles would seem to render its importance as a buffer problematical were it not for the fact that there is present in the corpuscles the relatively great store of organic phosphorus which has been found to be chemically unstable—sensitive to acids and probably also to enzymes present in the cells—and which therefore may be available in case of need. Further investigation of some of the phases of this problem is being carried on.

#### SUMMARY.

On the basis of the results reported above phosphoric acid compounds found in human blood may be divided into two classes: (1) the acid-soluble—soluble in dilute acids and precipitated with the proteins by alcohol-ether,—and (2) the lipoid-phosphoric acid compounds—soluble in alcohol-ether and precipitated with the proteins by dilute acids. The two groups are apparently sharply defined and since, in general, their sum is equal to the total phosphates, the presence of other forms of phosphoric acid combination in blood in significant amounts is doubtful.

In the second of these groups are contained substances of the type of lecithin, in the first group, inorganic phosphates and an unknown compound (or compounds) which is decomposed by heating with acids yielding phosphoric acid.

The amount of the unknown form of phosphoric acid combination in plasma is relatively small—up to 10 per cent of the total phosphates—while in the corpuscles it composes 60 to 80 per cent of the total phosphate. The significance of this compound is discussed.

The corpuscles are relatively richer in all types of compound than the plasma and there is also considerably less variation in their composition in different individuals than is the case with the plasma.

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## CHOLESTEROL IN MILK.

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(Received for publication, August 7, 1918.)

The advent of rapid and relatively accurate micro methods for the determination of cholesterol has furnished the stimulus for numerous investigations on the cholesterol content of human blood in health and in disease, and of the blood of animals under various experimental conditions.<sup>1</sup> As yet these investigations have, however, been extended in a few cases only to the study of body fluids other than blood. This is particularly true in the case of milk, as in a recent search through the literature we were able to find only a few isolated determinations of cholesterol in this fluid.

In view of this scarcity of published data we have felt it worth while to place on record the results of a series of determinations of cholesterol in milk which have been made in connection with a study of the non-protein constituents of this liquid now in progress in this laboratory.

The samples of cow's milk were personally collected by one of us at the Hospital dairy, and were examined within 2 hours after collection. For the samples of human milk we are indebted to Dr. Fritz Talbot. These specimens were all secured from normal women, and were, like the specimens of cow's milk, examined when fresh.

• The determinations of cholesterol were made by Bloor's colorimetric method. For the determination of fat we have used the Babcock method for all the specimens of cow's milk, while with the specimens of human milk Bloor's nephelometric method<sup>2</sup> was employed.

<sup>1</sup> Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

<sup>2</sup> Bloor, *J. Am. Chem. Soc.*, 1914, xxxvi, 1300.

The results presented in Table I would seem to show a direct and proportional variation of the cholesterol with the total fat content of cow's milk. In the case of the results on human milk given in Table II, this proportionality is to be noted, but many exceptions occur.

The effect of the cholesterol content of the food on blood cholesterol has been repeatedly demonstrated. That the ingestion of cholesterol increases the content of this substance in the milk seems probable, although experimental proof of this point is lacking.

TABLE I.  
*Cholesterol in Cow's Milk.*

Cow No.	Cholesterol.	Fat.
	<i>mg. per 100 cc.</i>	<i>per cent</i>
22	17.6	5.0
10	17.6	4.6
4	16.4	4.4
59	16.0	4.2
7	15.8	4.7
32	15.6	3.4
45	15.2	5.3
33	15.2	3.4
42	14.4	4.0
44	14.0	3.9
55	13.6	3.3
49	12.8	4.5
2	12.0	3.7
*A	11.2	3.4
16	10.5	3.2

We would suggest, therefore, that the exceedingly regular results obtained by us on cow's milk in distinction to the variations occurring in our series of human milks, are perhaps due to the fact that these samples of cow's milk were obtained from a single dairy, and from animals fed on exactly the same ration. Our samples of human milk were on the other hand taken from women living under a great variety of conditions, and with a corresponding lack of uniformity in food intake.

The distinctly higher level of our series of human milks may perhaps also be attributed to the higher cholesterol content of the food of nursing mothers as compared to that of cows.

TABLE II.  
*Cholesterol in Human Milk.*

Mother No.	Cholesterol.	Fat.	Age of child.
	<i>mg. per 100 cc.</i>	<i>per cent</i>	<i>days</i>
96	38.0	5.4	330
87	31.2	6.8	—
120	28.9	8.0	330
113	28.0	7.2	30
121	28.0	8.0	330
123	26.5	3.8	90
63	26.4	5.2	—
89	26.2	6.6	60
139	24.4	5.5	60
92	24.4	8.0	42
82	23.5	7.1	35
93	23.5	5.4	63
95	23.5	2.5	330
37	23.0	—	21
133	23.0	5.5	275
81	22.4	5.6	30
23	22.2	6.8	14
117	21.2	2.7	150
110	20.0	—	240
161	20.0	4.0	14
97	19.3	2.7	330
158	18.8	2.0	180
22	17.8	5.2	14
157	17.6	3.6	28
112	17.6	4.2	30
98	17.6	5.4	330
36	16.8	1.8	21
91	16.4	4.5	28
138	16.4	2.9	60
80	16.0	1.2	120
79	16.0	2.1	120
88	16.0	3.2	49
163	14.8	2.4	24
126	14.8	1.4	29
155	14.4	2.0	25
156	13.9	2.6	28
116	13.6	2.2	210
154	13.6	1.6	25
76	12.9	1.5	5
120	12.8	1.5	330
124	12.4	0.8	56
127	11.0	0.7	35
137	10.0	1.2	—
85	9.6	2.3	—



## VITAMINE STUDIES. I.

### OBSERVATIONS ON THE CATALASE ACTIVITY OF TISSUES IN AVIAN POLYNEURITIS.\*

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(Received for publication, July 26, 1918.)

Since Loew's observation (1) that the decomposition of hydrogen peroxide by plant and animal tissues is due to a special enzyme, to which he gave the name "catalase," much has been written concerning the functions of this enzyme. Of the later investigators, Battelli and Stern (2) are among the first to make quantitative measurements of the catalytic activity of various animal tissues by means of hydrogen peroxide. These authors found that the liver contained the largest amount of catalase while the brain contained the least. Further, these investigators found that a warm-blooded animal, such as the guinea pig, possessed tissues which were more highly catalytic to hydrogen peroxide than those of the frog.

At about the same time Jolles (3) also observed that the tissues of warm-blooded animals were higher in catalase content than those of the cold-blooded animals, represented in their work by the fish. These observers also studied the catalase activity of blood under normal and pathological conditions. It was found that the catalytic activity of blood was almost 50 per cent below normal in tuberculous patients. Similarly, the catalase value fell to a low level in nephritis, while in carcinomatous conditions the activity was practically lost.

As early as 1901, Cotton (4) showed that it was possible to differentiate between blood from different sources by measuring the quantity of oxygen liberated per unit of time by a definite amount of defibrinated blood. In support of this he showed that the blood of man had a value of 500 to 600 cc., the horse and pig 300 to 350 cc., ox 165 to 170 cc., guinea pig 115 to 125 cc., and sheep 60 to 65 cc. Kastle and Loevenhart (5) have expressed the opinion that catalase plays a physiological rôle in cell oxidation, although they believe (6) that the power of catalase to decompose hydrogen peroxide

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\* Published with the approval of the Director, as Paper 129, Journal Series of Minnesota Agricultural Experiment Station.



is not necessarily a physiological function. Battelli and Stern (7) and Buxton and Shaffer (8) have found that the catalytic activity of embryonic tissue is lower than that of adult tissue. Similar work by Mendel and Leavenworth (9) revealed similar results, and they also recorded the fact that the liver and kidney tissues were highest in catalase activity. Ostwald (10) showed that the sperm contains more catalase than does the ovarian substance, and Liebermann (11) found relatively large quantities in blood-free adipose tissue.

In his studies on fasting animals, Hawk (12) has observed that fasting tends to lower the catalytic power of tissues, and found that the animal was able to establish a sort of immunity or resistance, having on the second fast, tissues which were higher in catalase than those of animals fasting for the first time. He found also that normal liver is the most active tissue and that kidney, spleen, lung, heart, muscle, brain, and pancreas, when arranged according to their catalase content, came in the order named. Opie and Barker (13) confirmed the work of Jolles (3) in regard to the lowered catalytic activity of tuberculous tissue while Winternitz and Meloy (14) could find no striking differences between normal tissues and those of a pneumonia patient. In the study of syphilitic tissue they found the catalase content very low. In diabetes mellitus these authors found no difference in kidney or liver catalase but found the lung and spleen to be lowered 30 and 15 per cent respectively.

It is reasonable to suppose that oxidative activity falls in certain diseased tissues for Kastle and Angoss (15) have shown that the peroxidase activity of blood of diseased patients ranges from 60 to 25 per cent of normal. In the last few years Burge and his coworkers have published a number of papers attempting to show that a definite relationship exists between the catalase content of tissue and the oxidative processes in metabolism. He has also shown that catalases may have a destructive action against autolytic enzymes (16). He contends that in disease the oxidative processes become hampered and fail to balance the autolytic changes. In support of this idea Jacoby (17), Riess (18), Welsch (19), and Burge (20) observed slowing up of oxidative changes and a hastening of autolytic processes in phosphorus poisoning. Schlesinger (21) also points out that tissues show a decided tendency toward autodigestion in diseases of the circulatory and respiratory systems. Burge has also shown that catalase is higher in active tissue than in tissue which has not been active (22), and that the breast muscle of the wild pigeon has a greater catalytic effect on hydrogen peroxide than similar tissue taken from the bantam chicken (23). These authors (24) also support the findings of Hawk (12) that inanition is accompanied by low catalytic activity. Kennedy and Burge (25) have found that pancreatectomy prevents catalase formation and conclude from this work that catalase is related to oxidative activity and that the extirpation of the pancreas prevents a hormone passing to the liver. Burge believes the liver to be the source of catalase and has shown that the catalytic activity of the blood falls during ether anesthesia but by electro-

lytic stimulation of the "liver distribution" of the splanchnic nerves he was able to show increased catalytic activity of hepatic blood (26). According to Burge (27), there is a relationship between catalase activity, acidosis, and normal oxidative processes (28). In 1916 Ramoino (29) published results of experiments showing that the respiratory quotient falls in avian polyneuritis, but when extracts of rice bran are added and the pigeons have started to recover, the respiratory quotient increases to normal.

Similarly, Funk and von Schönborn (30) are of the opinion that vitamins have a distinct effect on carbohydrate metabolism. It is common knowledge that all pigeons do not react the same to diets composed solely of polished rice and the hypothesis has been advanced by Vedder and Clark (31) that fowls with high metabolic activity require larger amounts of vitamins and succumb most promptly to diets of polished rice. Other writers (32, 33) have observed that there is a decided tendency to hasten the onset of polyneuritis in pigeons by increasing the fuel value of the dietary by additions of vitamin-free materials, such as glucose or starch.

#### INTRODUCTION.

In view of the fact that the above evidence seems to indicate that the enzyme, catalase, is correlated with physiological processes of an oxidative nature and also that the physiological rôle of the vitamin, which exhibits a curative action in polyneuritis, is probably related to the oxidative processes, it was thought that a study of the catalase content of tissues of polyneuritic pigeons might be of interest.

#### *Experimental Feeding.*

Six pigeons were placed on a diet of polished rice and six on an ordinary diet of corn, wheat, and oats. Sand was fed every other day to both sets of birds. The pigeons were weighed every 3 days, on the average, and later in the experiment, a number of temperature records were obtained by placing a thermometer in the pigeon's crop. The water-soluble vitamin<sup>1</sup> was obtained by extracting wheat embryo with dilute alcohol and evaporating this extract upon dextrin. It has been proved by McCollum and Kennedy (34) and substantiated by Drummond (35) that the curative properties of various cereals, leaves, wheat embryo, corn germ, potato juice, etc., are due, in all probability, to an

<sup>1</sup> I wish to thank Miss Cornelia Kennedy for the preparation of the vitamin extract.

antineuritic substance, soluble in water and alcohol, which has been given the name water-soluble B.

As soon as the birds were killed, samples of blood were taken by absorbing approximately 1 gm. of blood in small strips of oxalated porous paper which had been weighed previously in ground glass weighing bottles. The bottles were closed as soon as the samples were taken and weighed immediately. The organs were isolated at once, washed free from blood with a 0.9 per cent solution of sodium chloride, and minced very fine, and approximately 1 gm. samples were placed in ground glass weighing bottles. With a few exceptions all minced tissue samples were run in duplicate while the blood samples were determined in triplicate.

#### *Determination of Catalase.*

The catalase content of tissues was determined by measuring the quantity of oxygen liberated from "Oakland Dioxygen," 3 per cent  $H_2O_2$ , in a 15 minute period by 1 gm. samples of tissue or blood. All determinations were made at  $20^\circ C$ . by suspending the hydrogen peroxide bottle in a constant temperature bath. In order that the samples might be agitated in a uniform manner the bottle was attached to a Van Slyke (36) pulley and motor which allowed a uniform agitation of 132 double shakes per minute of the hydrogen peroxide bottle as it hung suspended in the water. The gas was allowed to replace water in inverted graduated cylinders, and corrections were made for temperature, and atmospheric and vapor pressures. All gas volumes were calculated to standard conditions; *i.e.*,  $0^\circ C$ . and 760 mm. pressure.

#### DISCUSSION.

The results of the measurements are summarized in Table I.

Three normal birds (Nos. 1, 2, and 12) were used in the preliminary work to obtain sufficient knowledge of the laboratory technique. These data, although agreeing with those of Pigeons 3, 11, and 7, are not included in the table as a few determinations were lost in perfecting the methods. Four pigeons (Nos. 6, 9, 4, and 10) became polyneuritic, while Pigeon 5 did not succumb to the disease and was killed at the end of a 47th day period of rice

feeding. Pigeon 4 became polynuritic on the 24th day after being placed on a diet of polished rice, Pigeon 6 on the 29th day, Pigeon 9 on the 43rd day, and Pigeon 10 on the 23rd day. Pigeon 5 was so active on the 47th day of rice feeding that it was thought to be of interest to make catalase measurements on its tissues. Pigeon 8 subsisted, for 55 days, on a diet composed solely of rice. On the 54th day this pigeon was active but was found dead the following day, due in all probability, to fasting. The catalase content of the tissues was found to be practically normal with the exception of the blood. The fall in catalase and the subsequent

TABLE I.

*Oxygen Liberated from Hydrogen Peroxide by 1 Gm. of Tissue in 15 Minutes at 20°C.*

Tissue.	Normal pigeons.				Polynuritic pigeons.			Polynuritic pigeons after receiving water-soluble B.			Rice-fed pigeon which was immune to polynuritis. No. 5
	No. 3	No. 11	No. 7	Average.	No. 6	No. 9	Average.	No. 4	No. 10	Average.	
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Liver.	2,758	2,835	2,170	2,588	1,250	1,205	1,227	2,690	2,870	2,780	2,604
Kidney.	985	1,497	840	1,109	523	506	514	1,193	1,072	1,132	916
Pancreas.	313	530	419	421	282	279	280	579	391	485	203
Heart	166	206	219	197	66	143	104	165	182	171	151
Breast muscle.	91	116	135	114	102	97	99	177	171	174	126
Lung.	88	183	103	125	51	54	53	57	153	105	168
Blood	24	64	Lost	44	16	5	11	19	29	24	2

rise are expressed in Table II in per cent of normal. Chart 1 shows that Pigeon 5 had lost nearly 30 per cent of its original body weight and yet its tissues were not appreciably below normal in catalytic activity. The point at which each bird was killed is marked by an X.

Chart 2 gives the comparative supplies of this oxidizing enzyme in the various tissues, as measured by the amount of oxygen liberated. From these data it would seem that the catalytic activity of tissues of pigeons, subsisting on a diet solely of rice, falls until it reaches its lowest level at the acute stage of polynuritis. It would also appear that the administration of water-soluble vitamin

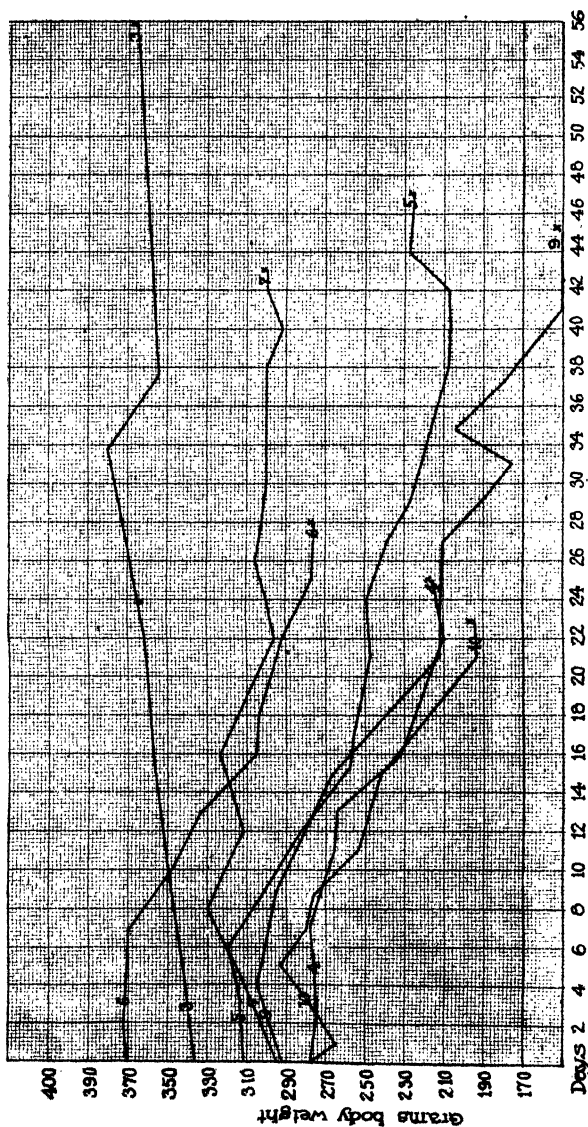


CHART 1. Curves showing weights of pigeons. Pigeon 11 is not included in this chart as it was killed early in the experiment.

stimulates directly or indirectly oxidative processes, if oxidative processes can be measured in terms of catalase.

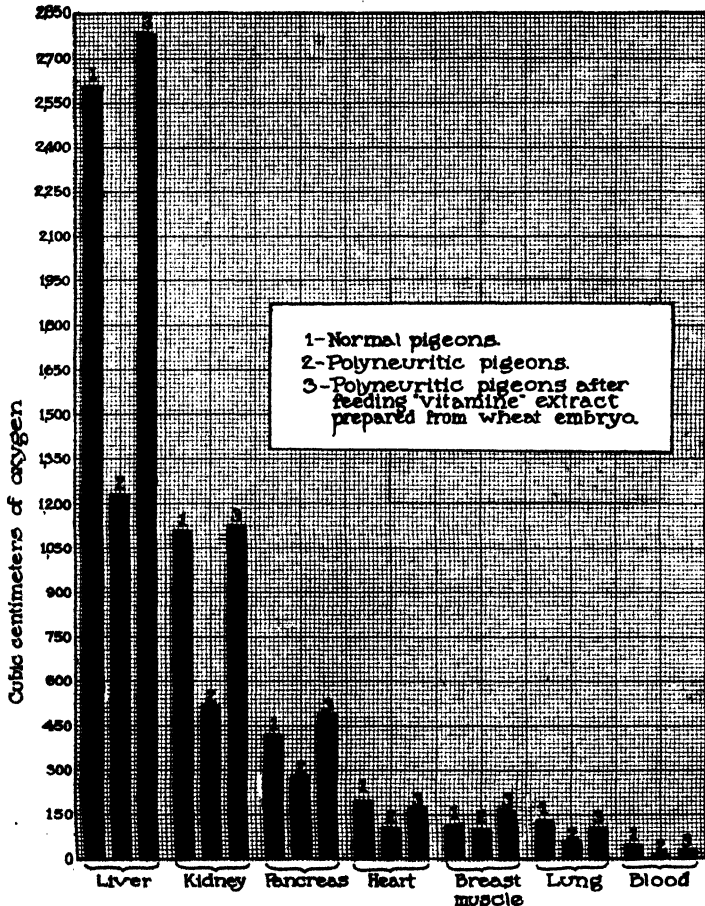


CHART 2. Comparative catalytic activity of various tissues.

This supports the work of Ramoino (29) who showed that the carbon dioxide output diminishes with the onset of the disease. It is of interest to point out that the order in which the tissues

group themselves as to catalase content is practically the same as the order in which these tissues fall when grouped according to their vitamine content. Cooper (37) has shown that dried tissues, when arranged in the order of their effectiveness in curing polyneuritis, fall in the order liver, brain, heart, and voluntary muscle. Similar conclusions have been published recently by Osborne and Mendel (38).

Pigeon 10 became polyneuritic on the 23rd day and was given water-soluble vitamine immediately. In 35 minutes the bird was attempting to walk and in 3 hours had practically recovered. At this point the bird was killed.

#### *Temperatures of Pigeons.*

Unfortunately no systematic records were taken of temperatures until the latter part of the experiments. Recent work on fourteen normal pigeons reveals an average temperature (by crop) of  $41.5^{\circ}\text{C}$ . In no case, where pigeons have received polished rice for 8 or 10 days, have we found temperatures exceeding  $39^{\circ}\text{C}$ . Pigeon 6 showed a temperature of  $36^{\circ}\text{C}$ . at the time of killing (in the early stages of polyneuritis). Pigeon 5, which subsisted for 47 days on polished rice without pronounced symptoms of polyneuritis, showed a temperature of  $37^{\circ}\text{C}$ . when killed. Pigeon 9, on the other hand, was dying when found and gave a temperature reading of  $28^{\circ}\text{C}$ ., or  $13.5^{\circ}\text{C}$ . below normal, which is equivalent to  $24.3^{\circ}\text{F}$ . below the normal temperature. Of course it must be recognized that this bird had lost over 48 per cent of its body weight, and these unusual conditions cannot be attributed alone to the lack of water-soluble vitamine. Although catalase activity has been shown to diminish in tissues of fasting animals (12), it is interesting to note that the tissues of Pigeon 5, which did not become polyneuritic, had about normal catalase content, although the bird had lost nearly one-third of its weight. Similar conditions exist in the case of polyneuritic pigeons which showed high catalase activity after administration of the vitamine extract although they had lost a relatively large proportion of their original body weight.

It should also be recorded at this point that on June 12, 1918, Pigeon 9, while on a rice diet, had a temperature of  $37.1^{\circ}\text{C}$ . While the cage was being cleaned, a cockroach was seen to enter

the cage and before it could be removed was eaten by the pigeon. The next morning the temperature had risen to 39.8°C. and the bird was very active. The effects of the insect were noticeable for 14 days at which time the pigeon again began to show the effect of rice feeding.

## SUMMARY.

Data are presented which show that the catalase content of tissues was lowered to the extent of 44.4 per cent (see Table II) in avian polyneuritis. Polyneuritic pigeons which had been given a water-alcohol extract of wheat embryo, containing water-soluble B, possessed tissues which were approximately normal

TABLE II.  
*Catalase Activity of Tissues.*

Tissue.	Polyneuritic pigeons. Per cent of normal.	Polyneuritic pigeons receiving water-soluble vitamine. Per cent of normal.
Liver.....	44	110
Kidney.....	53	102
Pancreas.....	33	115
Heart.....	34	86
Breast.....	13	152
Lung.....	57	84
Blood.....	75	56
Average.....	<b>44</b>	<b>101</b>

(101.2 per cent) in catalase content. One pigeon in the early stages of polyneuritis showed improvement and increase in body temperature after eating a cockroach which accidentally obtained access to the cage, which would indicate the presence of vitamine in the body of insects. Body temperatures which are about 41.5° C. in normal pigeons were found to be lowered appreciably in avian polyneuritis. It is probable that polyneuritis is accompanied by incomplete or partial oxidation, with the accumulation in the tissues of products of incomplete oxidation. It is also probable that water-soluble vitamins function, directly or indirectly, in the stimulation of oxidative processes, thereby clearing the tissues of toxic materials. When pigeon tissues are arranged in the order of their catalase content (as measured by



the oxygen liberated from hydrogen peroxide), the tissues group themselves in the order of their metabolic activity and also in the order of their content of water-soluble vitamine.

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## EPIMERIC HEXOSAMINIC ACIDS.

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(Received for publication, July 29, 1918.)

The synthesis of  $\alpha$ -hexosaminic acids had for its principal aim the allocation of the  $\text{NH}_2$  group in the natural and synthetic aminohexoses.

Irvine and Hynd<sup>1</sup> have shown that depending on conditions glucosamine may be converted either into glucose or into mannose. Levene and LaForge have shown that choosing the proper conditions one may obtain from glucosamine either anhydrogluconic or anhydromannonic, either anhydrosaccharic or anhydromannosaccharic acids. Hence there was no foundation for the presence for either one of the two alternative configurations for glucosamine.

Since at present for the purpose of proving the configuration of the amino sugars there exists no direct chemical means such as Fischer and his coworkers employed for developing the structure of nitrogen-free sugars, one has to resort to methods of analogy.

It was hoped that a comparison of the properties of pairs of epimeric hexonic acids with corresponding pairs of hexosaminic acids might offer some suggestions for the allocation of the amino group. The properties which are compared in the present discussion are: first, the equilibrium of the two epimers formed on the synthesis of the hexonic acids from the corresponding pentoses; second, the direction of the rotation of the  $\alpha$ -carbon atom in corresponding pairs of epimers. An attempt to compare these properties had already been made by the present writer at a time when pairs of epimeric hexosaminic acids had not yet been known.

<sup>1</sup> Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1912, ci, 1128; 1914, cv, 698. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 351.

In the present communication the properties of three pairs of epimeric  $\alpha$ -hexosaminic acid are compared. Designated by the parent pentose the substances are: two arabinohexosaminic acids, two lyxohexosaminic acids, and two xylohexosaminic acids. Of the first pair one can be prepared from glucosamine, occurring in nature, or synthetically from arabinose. The substance employed in the present investigation was prepared by oxidation of glucosamine. The epimeric acid as prepared by the action of pyridine on glucosaminic acid.

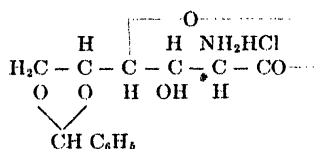
Of the second pair also, one may be obtained from the naturally occurring sugar chondrosamine as well as by synthesis from lyxose. In the present work the substance was obtained by both methods. The acid epimeric to chondrosaminic was prepared synthetically. The third pair was prepared synthetically from xylose.

Both the pyridine and the synthetic method always furnished a mixture of the two epimeric substances and in every substance the separation was accomplished by means of fractional crystallization either out of water or out of dilute ethyl or methyl alcohol.

It was fortunate that one member was obtainable in a condition of undisputed purity in every one of the three pairs. The purity of glucosaminic and chitosaminic acids is to be accepted by reason of their preparation from naturally occurring sugars. One, the dextroxylohexosaminic acid, forms a crystalline lactone and this was converted into a pure acid. Also, fortunately it was possible to obtain epiglucosaminic acid in a state of unquestionable purity. Under certain conditions out of a mixture of the two only one namely the epiglucosaminic acid, formed a crystalline lactone.

Data obtained on this pair of epimers was of great assistance in the separation of the individual members of the other two pairs; namely, on this pair the numerical value of the optical rotation of the  $\alpha$ -carbon atoms was established. These values were then employed as a standard of purity in the separation of the individual members of the other two pairs. Indeed, when this value was obtained further recrystallization remained without influence on the rotation of the amino-acid.

Later in the work the levoxylohexosaminic acid was obtained from a crystalline derivative of the acid; namely, from the benzal xylohexosaminic lactone hydrochloride.



This substance yielded an acid possessing the same optical rotation as the parent substance. Thus by fractional crystallization alone the complete separation of the epimers was accomplished.

The identity of the individual amino-acids was further demonstrated by results of deaminization experiments. These results will be communicated in a separate publication.

Turning then to the principal point of the investigation, namely to the comparison of the properties of epimeric amino-acids amongst themselves and with the corresponding members of non-nitrogenous sugar acids, we find that in each pair of hexosaminic as on each pair of hexonic acid the rotation of the  $\alpha$ -carbon atom of one member is to the right and the other to the left.

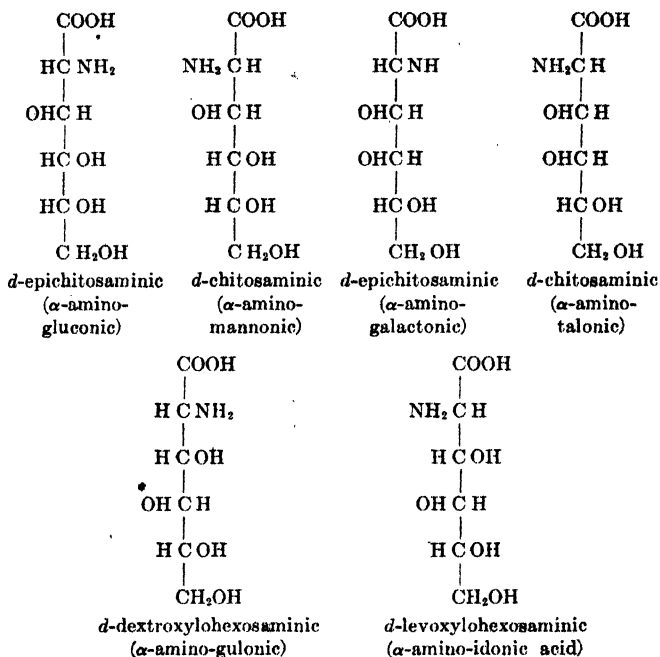
Incidentally it is to be noted that the numerical value of the rotation of the  $\alpha$ -carbon in all six hexosaminic acids is practically constant. In the series of hexonic acids those rotating to the right are gluconic, galactonic, gulonic.

Turning their attention towards the equilibrium of the products of condensation of pentoses with prussic acid,<sup>2</sup> Fischer and his co-workers found that arabinose yields in preponderance mannonic acid, which is levorotary; lyxose and xylose yield galactonic and gulonic acid, respectively, which are dextrorotary.

In the action of prussic acid on amino-pentosides, arabinose yields principally the levorotary acid, lyxosamine and xylosamine both yield in preponderance a dextrorotary acid. Thus there seems to exist a complete analogy in the predominating products formed from the pentoses of those from the amino-pentosides, from the view point of the properties of their  $\alpha$ -carbon atoms. May not, therefore, there exist also a similarity in their configuration? For the present this is only a suggestion. If this view should find further corroboration then the configuration of the six known hexosaminic acids will be expressed as follows:

<sup>2</sup> Fischer, E., *Ber. chem. Ges.*, 1890, xxiii, 2611, 2625. Fischer, E. and Bromberg, O., *ibid.*, 1896, xxix, 581.

## Epimeric Hexosaminic Acids



In a previous publication<sup>3</sup> identical conclusions were reached in regard to chitosaminic and chondrosaminic acids, whereas in regard to the derivatives of xylose the conclusion was contradictory and was evidently erroneous. The fact that in the earlier experiment after the action of pyridine an acid was obtained with a higher dextrorotation than the original is explained simply by the fact that in the presence of impurities only the more insoluble substances crystallized, while the more soluble remained in the mother liquor.

It would seem more rational to abandon the term glucosamine and glucosaminic acid and to return to the older terms chitosamine and chitosaminic acid.

The respective optical rotations and the numerical values of the rotations of the  $\alpha$ -carbon atoms are as follows:

<sup>3</sup> Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145; Levene, P. A., and Meyer, G. M., *ibid.*, 1916, xxvi, 355; 1917, xxxi, 627.

Acid.	$[\alpha]_D^{20}$	B-A	$\frac{B-A}{2}$
A <i>d</i> -Chitosaminic.....	-15	25	12.5
B <i>d</i> -Epichitosaminic.....	+10		
A <i>d</i> -Chondrosaminic.....	-17	25	12.5
B <i>d</i> -Epichondrosaminic.....	+8		
A <i>d</i> -Levoxylohexosaminic.....	-11	25	12.5
B <i>d</i> -Dextroxylohexosaminic.....	+14		

This table contains the best evidence as yet advanced in support of Van't Hoff's optical superposition theory. In the series of  $\alpha$ -hexosaminic acids the law holds true not only in regard to the direction of the rotation but also in regard to its numerical value. The reason for the more perfect agreement between the numerical values in this series of substances may be attributed to the fact that the phenomena of electrolytic dissociation here play a comparatively subordinate part, and that individual amino sugar acids are accessible in purer state, since the separation of their epimers is more perfect than those of the ordinary sugar acids.

#### EXPERIMENTAL.

##### *Epichitosaminic Lactone Hydrochloride.*

100 gm. of chitosaminic acid are taken up in 1,000 cc. of water to which 100 gm. of pyridine are added and heated in an autoclave at 105°C. for 4 hours. The solution is then evaporated under diminished pressure to a small volume, so that the greater part of chitosaminic acid crystallizes in the distillation flask. The residue is again brought into solution with a minimum amount of boiling water. To the solution while still hot an equal volume of 95 per cent alcohol is added. The solution then becomes filled with crystals of chitosaminic acid. To complete the crystallization the material is allowed to stand from 8 to 12 hours.

The filtrate is then concentrated under diminished pressure (the temperature of the water bath should not exceed 50°C.) to a small volume. To this, hot 95 per cent alcohol is added until the solution begins to show opalescence, and enough hot water is then added to clarify the solution. On standing, more readily after some scratching, a crystalline substance begins to deposit. The deposit reaches its maximum in about 24 hours. This substance is dissolved in a minimum amount of hot water, boiled with charcoal, and the filtrate treated with 95 per cent alcohol in the manner just described. The crystalline substance obtained is at times nearly colorless, and at times of very light tan. It consists of a mixture of varying proportions of chitosaminic and epichitosaminic acids. On one occasion it consisted of pure chitosaminic acid. The optical rotation of the substance varied from  $[\alpha]_D^{20} = +3.0^\circ$  to  $+8.0^\circ$ , and on one occasion it had  $[\alpha]_D^{20} = +10.0^\circ$ .

For further purification the epichitosaminic acid was converted into its lactone. This was accomplished in the following way: to 3 gm. of the acid 2 cc. of 99.5 per cent alcohol and 1 cc. of benzaldehyde were added, and dry hydrochloric acid gas passed. The acid at first goes into solution and subsequently a crystalline deposit begins to reappear. The treatment with the gas is continued approximately 7 minutes. After standing 8 to 12 hours, 3 cc. of dry ether (dried over sodium) were added, and the material was allowed to stand an additional 12 or 24 hours in order to obtain the maximum yield of the lactone hydrochloride. The addition of benzaldehyde has for its purpose the conversion of the chitosaminic acid into the benzal of its ethyl ester. Under these conditions it remains in solution and permits the crystallization of the epichitosaminic acid lactone. In the absence of benzaldehyde an amorphous precipitate is formed which probably consists of a mixture of the two lactones.

The lactone is crystallized out of methyl alcohol. For this purpose it is advisable to dissolve the crude substance in a large excess of hot methyl alcohol, decolorize the solution with charcoal, and then concentrate it to a small volume. The lactone crystallizes in form of colorless prismatic needles. The substance had a melting point = 203°C. (with gas evolution). Its composition was as follows:

0.1040 gm. substance gave 0.1286 gm.  $\text{CO}_2$  and 0.1040 gm.  $\text{H}_2\text{O}$ .  
 0.100 " " required for neutralization 4.65 cc. 0.1 N acid.  
 0.100 " " " 4.50 cc. 0.1 N  $\text{AgNO}_3$ .

	Calculated for $\text{C}_6\text{H}_{12}\text{NO}_5\text{Cl}$ :	Found:
C.....	33.71	33.72
H.....	5.67	5.47
N.....	6.55	6.51
Cl.....	16.25	15.97

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.90 \times 100}{1 \times 2} = +45.0^\circ$$

*Epichitosaminic Acid.*

2 gm. of the lactone were dissolved in 20 cc. of distilled water and the solution was rendered alkaline by means of a solution of barium hydroxide. The solution was allowed to stand over night, then the barium was removed quantitatively by means of sulfuric acid, the hydrochloric acid by silver carbonate, and the excess of the latter reagent by hydrogen sulfide. The mother liquor of the silver sulfide was concentrated to a small volume; on addition of little alcohol the epichondrosaminic acid crystallizes immediately in prismatic needles. The substance had a melting point of  $198^\circ\text{C}$ . (uncorrected) with gas evolution.

The composition of the substance was the following.

0.0992 gm. substance gave 0.1336 gm.  $\text{CO}_2$  and 0.0606 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{NO}_5$ :	Found:
C.....	36.92	36.80
H.....	6.66	6.72

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{\text{Initial. } +0.20 \times 100}{1 \times 2} = +10^\circ \quad [\alpha]_D^{20} = \frac{\text{Equilibrium. } +0.78 \times 100}{1 \times 2} = +39^\circ$$

The rotation of chitosaminic acid being  $-15.02^\circ\text{C}$ ., that of the  $\alpha$ -carbon atom is  $\frac{10.0 - (-15.02)}{2} = 12.51^\circ\text{C}$ .



*Synthesis of Amino Sugar Acids.*<sup>1</sup>

In the earlier work of Levene and LaForge the conditions given by Fischer and Leuchs for the synthesis of chitosamine were followed. In the subsequent work, however, it was found that the reaction was very capricious and the results fluctuating. Thus for a long time the maximum yield of xylohexosaminic acid was 10 per cent, and a certain number of experiments ended in complete failure. However, after some experimentation it was discovered that the result of the reaction of prussic acid on amino-pentosides was determined on one hand by the temperature, and on the other hand on the duration of the reaction. The optimum time and temperature vary for every individual sugar, and have to be determined empirically.

When the optimum conditions were discovered the yield of xylohexosaminic acid rose from 10 per cent to 50 per cent. True, the reaction is so sensitive that often in spite of the best care the optimum conditions are missed and the yield falls from 50 to 30 or 25 per cent of the employed amino-pentoside.

*Chondrosaminic and Epichondrosaminic Acids.*

The conditions of the synthesis of lyxohexosaminic acid are the following. 60 gm. of amino-lyxoside dried first under diminished pressure over soda lime and subsequently in a vacuum desiccator at 50°C. were taken up in 80 cc. of water. To the suspension 36 cc. of an 80 per cent solution of prussic acid in water and 5 cc. of ammonium water were added. The flask, provided with a thermometer, was immersed in a warm water bath and the temperature maintained between 37–40°C. As soon as the temperature showed a slight tendency to rise the flask was immediately transferred to a cold water bath until the temperature fell to 37°C. The amino-pentoside rapidly dissolved, giving rise to a slightly brown solution which gradually turned dark and quite viscous. After a certain depth of color and a certain viscosity are obtained, which one learns to recognize by experience, the reaction is interrupted. Generally it requires about 15 minutes to complete the reaction. The flask is then

<sup>1</sup> Levene and La Forge, *J. Biol. Chem.*, 1915, xxi, 351; xxii, 331.

immersed into a cooling mixture and the temperature of the solution is allowed to fall to  $0^{\circ}\text{C}.$ , and is poured into 300 cc. of concentrated hydrochloric acid previously cooled to the same temperature. The solution is then saturated with hydrochloric acid gas and allowed to stand not less than 1 hour; it may be allowed to stand over night. The solution is concentrated under diminished pressure at a temperature of the water bath not exceeding  $75^{\circ}\text{C}.$  In course of the concentration considerable ammonium chloride crystallizes out in the flask. When the solution becomes quite viscous the concentration is interrupted and the contents of the flask are poured into 2 liters of 95 per cent alcohol. Generally a small quantity of tar separates out which is removed by filtration through cotton wool. The alcoholic filtrate is concentrated under diminished pressure, the residue is dissolved in about 1 liter of water, and 200 gm. of moist barium hydroxide are added and the mixture is repeatedly evaporated to nearly dryness, the water being renewed at this phase. When all ammonium is removed the evaporation is interrupted and the barium is removed quantitatively by means of sulfuric acid, the hydrochloric acid by means of lead and silver carbonate. The final solution is concentrated under diminished pressure to a volume of about 50 cc. To this solution hot methyl alcohol is added gradually in small portions, avoiding an excess which causes precipitation of gum. On scratching, the  $\alpha$ -aminohexonic acids begin to crystallize immediately and crystallization is completed in 24 hours.

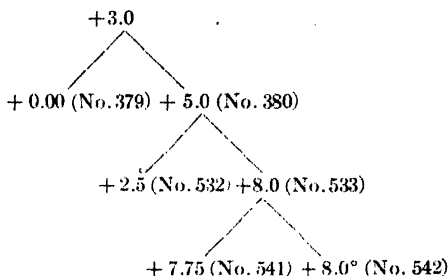
The specific rotation of the crude material was  $[\alpha]_D^{20} = +3.0$ . Since the specific rotation of chondrosaminic acid is  $[\alpha]_D^{20} = -17.0$  it is evident that the material is a mixture of the two epimers.

#### *Separation of the Epimers.*

250 gm. of mixed acid having  $[\alpha]_D^{20} = +3.0^{\circ}$  were dissolved in 750 cc. of hot water and allowed to stand over night at room temperature. By far the greater part of the material crystallized out. The rotation of this substance (No. 379) was  $[\alpha]_D^{20} = 0.00$ . The mother liquor was concentrated under diminished pressure to dryness and the residue again crystallized out of a minimum amount of water. A crystalline deposit formed on standing over

night (No. 532) with rotation  $[\alpha]_D^{20} = +2.5$ . The mother liquor was concentrated to dryness, taken up in a minimum amount of water and hot methyl alcohol was added to opalescence. Soon a crystalline deposit formed (No. 533) which had the rotation  $[\alpha]_D^{20} = +8.0$ . This material was again dissolved in a minimum amount of hot water and allowed to stand at room temperature over night. A crystalline deposit formed (No. 541) with the rotation of  $[\alpha]_D^{20} = +7.75$ . The mother liquor was concentrated under diminished pressure to dryness. The residue was taken up in a minimum amount of water and hot methyl alcohol was added until the appearance of a slight opalescence; the solution soon turned into a crystalline mass. The substance had the rotation  $[\alpha]_D^{20} = +8.0^\circ$ , thus showing that the final treatment did not accomplish any further fractionation.

The progress of fractionation is represented in the following diagram.



The substance with the rotation  $[\alpha]_D^{20} = 0.00$  was continually recrystallized out of water until a fraction was obtained with a rotation  $[\alpha]_D^{20} = -17.5^\circ$ .

The dextro epimer had the following composition.

0.1004 gm. substance gave 0.1358 gm.  $\text{CO}_2$  and 0.0624 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{13}\text{NO}_6$	Found:
C.....	36.92	36.89
H.....	6.66	6.96

The melting point was  $206^\circ$  (uncorrected) with gas evolution. The optical rotation was as follows:

$$[\alpha]_D^{20} = \frac{+0.16 \times 100}{1 \times 2} = +8^\circ$$

The rotation of chondrosaminic acid being  $-17.0$  and that of the epimer  $+8.0^\circ$ , the numerical value of the  $\alpha$ -carbon atom is  $\frac{8 - (-17.0)}{2} = 12.5^\circ$ .

*Xylohexosaminic Acid.*

Xylohexosaminic acid, as prepared previously by Levene and LaForge, consisted of a mixture of the two epimers, in which the dextro acid was in preponderance. At that time, this was not realized, and the substance was considered the pure dextro acid. Since some theoretical deductions were based on the earlier conception, it became necessary to repeat the earlier work on the pure epimer.

*Syntheses of d-Xylohexosaminic Acid.*

The general plan of the preparation of the material is as outlined in the paragraph on the preparation of lyxohexosaminic acids. The points of difference were the temperature of the reaction and the duration of the interaction of prussic acid and the amino-pentoside. The temperature was not allowed to exceed  $35^\circ\text{C}$ ., and the experiment was continued for 30 minutes. The further treatment was identical with that for the preparation of lyxohexosaminic acids.

*Separation of the Epimers.*—280 gm. of the mixed acids were dissolved in a minimum amount of water and methyl alcohol was added until a crystalline precipitate began to form. This was filtered off. It had a rotation of  $[\alpha]_D^{20} = +5.0$ . The mother liquor was concentrated to dryness under diminished pressure. The residue was then taken up in a minimum amount of water and on addition of alcohol a precipitate formed, which was removed by filtration and had the rotation  $[\alpha]_D^{20} = +2.00$  (No. 615).

180 gm. of this substance were dissolved in 300 cc. of hot water, and the solution was allowed to cool. A precipitate (No. 630) thus formed had a rotation  $[\alpha]_D^{20} = -1.0$ . 110.0 gm. of No. 630 were dissolved in 400 cc. of hot water and the solution was allowed to stand over night and a crystalline deposit (No. 641) formed

having a rotation  $[\alpha]_D^{20} = -1.0^\circ$ . On further fractionation of No. 641 the rotation of the substance did not change. The substance melted at  $200^\circ\text{C}$ . (uncorrected) with gas evolution. The composition was the following.

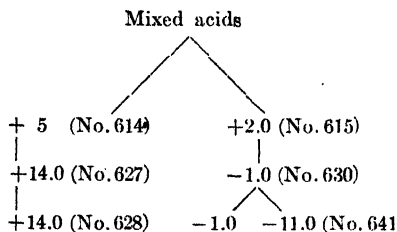
0.1000 gm. substance gave 0.1354 gm.  $\text{CO}_2$  and 0.0590 gm.  $\text{H}_2\text{O}$ .  
 0.1000 " " required for neutralization 5.25 cc. of N acid.

	Calculated for $\text{C}_6\text{H}_{12}\text{NO}_6$	Found:
C.....	36.92	36.92
H.....	6.66	6.60
N.....	7.18	7.35

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{\text{Initial. } -0.22 \times 100}{1 \times 2} = -11.0 \quad , \quad [\alpha]_D^{20} = \frac{\text{Equilibrium. } -0.63 \times 100}{1 \times 2} = -31.5$$

The progress of fractionation of the epimers is as follows:



The substance with rotation  $[\alpha]_D^{20} = +5^\circ$  was dissolved in a minimum amount of boiling water, allowed to stand 30 minutes, and while still warm was filtered. The precipitate thus formed had the rotation  $[\alpha]_D^{20} = +14.0^\circ$ . The yield of the substance was 87 gm. These were dissolved in 400 cc. of boiling water. On cooling, these formed a precipitate which was removed by filtration. The mother liquor was concentrated to dryness under diminished pressure. The residue was taken up in a minimum amount of water and hot methyl alcohol was added to opalescence. The crystallization began immediately. The optical rotation of both substances was identical:  $[\alpha]_D^{20} = +14.0^\circ$ .

Thus the optical rotation of the two epimers obtained by fractional crystallization were  $+14.0$  and  $-11.0$  respectively, and the

rotation of the  $\alpha$ -carbon atom is therefore  $\frac{A-B}{2} = \frac{14+11}{2} = 12.5$ .

The purity of the two epimers was ascertained by the conversion of the dextroisomer into its lactone hydrochloride, and the conversion of the levoepimer into the benzal lactone hydrochloride. Both lactones were subsequently converted into their parent acids.

*d-Dextroxylohexosaminic Lactone Hydrochloride.*

The lactone was prepared in the manner described in the following publication.<sup>5</sup> It was recrystallized from an excess of methyl alcohol. The substance had the melting point of 205°C. (uncorrected) with gas evolution. The substance had the following composition.

0.1000 gm. substance gave 0.1234 gm. CO<sub>2</sub> and 0.0504 gm. H<sub>2</sub>O.  
 0.2000 " required for neutralization 9.31 cc. 0.1 N acid.  
 0.1000 " " " titration of HCl 4.5 cc. N AgNO<sub>3</sub>.

	Calculated for C <sub>6</sub> H <sub>13</sub> NO <sub>3</sub> Cl:	Found:
C.....	33.71	33.65
H.....	5.67	5.64
N.....	6.55	6.52
Cl.....	16.25	15.98

*Conversion of the Lactone Hydrochloride into the Parent Acid.*—5 gm. of the lactone were dissolved in 50 cc. of water. An excess of barium hydroxide was added, and the solution was allowed to stand over night. The barium and the chlorine were then removed quantitatively. The filtrate was concentrated, and the acid crystallized on the addition of hot methyl alcohol. The substance had the melting point of 224° (uncorrected) with gas evolution. The substance had the following composition.

0.1070 gm. substance gave 0.1372 gm. CO<sub>2</sub> and 0.0620 gm. H<sub>2</sub>O.  
 0.010 " " " 1.27 cc. nitrogen in the Van Slyke micro-apparatus at 26°C. and 759 mm. pressure.

	Calculated for C <sub>6</sub> H <sub>13</sub> NO <sub>3</sub> :	Found:
C.....	36.92	37.04
H.....	6.66	6.86
N.....	7.18	6.99

<sup>5</sup> Levene, *J. Biol. Chem.*, 1918, xxxvi, 89.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{+ 0.28 \times 100}{1 \times 2} = + 14.0$$

*Monobenzal d-Levoxylohexosaminic Lactone Hydrochloride.*

2.5 gm. of the *d*-levohexosaminic acid were taken up in a solution of 15 cc. of 99.5 per cent of alcohol and 3 cc. of benzaldehyde. Hydrochloric acid gas was passed through the solution. The amino-acid went gradually into solution and after some time the entire solution solidified instantaneously. After 1 hour 2 cc. of ether (dried over sodium) were added and the material was allowed to stand an additional 2 hours. The lactone was then filtered and recrystallized out of methyl alcohol. The melting point was 206°C. (uncorrected) with gas evolution.

0.1023 gm. substance gave 1938 gm. CO<sub>2</sub> and 0.0568 gm. H<sub>2</sub>O.

0.1000 " " required for neutralization 3.34 N acid.

	Calculated for C <sub>13</sub> H <sub>15</sub> NO <sub>3</sub> ·HCl (molecular wt. 301.6):	Found:
C.....	51.72	51.81
H.....	5.35	5.57
N.....	4.69	4.68

The optical rotation of the substance in 50 per cent alcohol was:

$$[\alpha]_D^{20} = \frac{- 1.21 \times 100}{1 \times 2} = - 60.5$$

*Conversion of the Lactone into the Free Acid.*—10 gm. of the dry monobenzal lactone hydrochloride were dissolved in 50 cc. of water, and to the solution were added 12.5 gm. of barium hydroxide containing 5.0 gm. of Ba. The solution was placed on a boiling water bath. It was soon noticed that on warming, the solution turned yellow and developed ammonia. The experiment was therefore interrupted after 7 minutes. The solution was then rendered acid with sulfuric acid. The filtrate from the barium sulfate precipitate was extracted repeatedly with ether, and the aqueous solution was freed quantitatively from sulfuric and hydrochloric acids. The filtrate concentrated and the amino-

acid crystallized on addition of hot methyl alcohol. The melting point of the acid was 200°C. (uncorrected) with gas evolution. The substance had the following composition.

0.1000 gm. substance gave 0.1354 gm. CO<sub>2</sub> and 0.600 gm. H<sub>2</sub>O.

0.010 " " in the Van Slyke micro-apparatus gave 1.36 cc. nitrogen at 35°C. and 762 mm. pressure.

	Calculated for C <sub>8</sub> H <sub>13</sub> NO <sub>6</sub> :	Found:
C.....	36.92	36.98
H : .....	6.66	6.71
N .....	7.18	7.16

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{1 \times 2} = \frac{-0.22 \times 100}{1 \times 2} = -11.0 \quad [\alpha]_D^{20} = \frac{\text{Equilibrium.}}{1 \times 2} = \frac{-0.60 \times 100}{1 \times 2} = -30.0$$

Thus the rotations of the epimers obtained from the lactones were +14.08 and -11.0 respectively, and the rotation of their  $\alpha$ -carbon atoms is  $\frac{A+B}{2} = \frac{14+11}{2} = 12.5$ .





# THE ACTION OF NITROUS ACID ON EPIMERIC HEXOSAMINIC ACIDS.

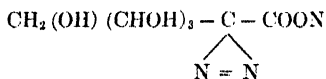
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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 30, 1918.)

It was definitely proven by the observation of Levene and La Forge,<sup>1</sup> that in course of the reaction of nitrous acid on chitosamine and on chitosaminic acid respectively, a Walden inversion takes place in one of the two substances. It was also shown<sup>2</sup> that under suitable conditions by the action of nitrous acid on the benzal of chitosaminic ethyl ester a diazo derivative is formed as an intermediary phase. Similarly, it was found by Levene and LaForge that chondrosamine and chondrosaminic acid, likewise xylohexosaminic acid and its lactone, gave rise to epimeric acids.

Accepting for the structure of the diazo compound the expression



it becomes evident that each one of a pair of epimers should on the basis of conventional theories lead to one and the same diazo derivative, and hence to one and the same deaminized acid. On the other hand, if two epimeric amino-acids, passing through the phase of the diazo derivative, give rise to two epimeric deaminized acids, one would be forced to accept the existence of two isomeric diazo bodies, perhaps of the nature of electromers. In view of these considerations it became important to obtain possession of epimeric amino-acids. Three such pairs have been prepared as reported in the preceding communication.<sup>3</sup> They are chitosaminic

<sup>1</sup> Levene, P. A., and LaForge, F. B., *J. Biol. Chem.*, 1915, xxi, 351.

<sup>2</sup> Levene and LaForge, *J. Biol. Chem.*, 1915, xxi, 345.

<sup>3</sup> Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 73.

and epichitosaminic; chondrosaminic and epichondrosaminic, *d*-dextroxylohexosaminic and *d*-levoxylohexosaminic acids. In each one of the three pairs the epimeric amino-acids gave rise to epimeric anhydro sugar acid as may be seen from the following tabulation.

A. Chitosaminic.....	anhydro- <i>d</i> -gulonic	acid.
Epichitosaminic.....	" - <i>d</i> -mannonic	"
B. Chondrosaminic.....	" - <i>d</i> -mucic	"
Epichondrosaminic.....	" - <i>d</i> -talomucic	"
C. Dextroxylohexosaminic.....	" - <i>d</i> -idosaccharic	"
<i>d</i> -Levoxylohexosaminic.....	" - <i>l</i> -saccharic	"

One peculiarity was observed in course of the work; namely, whereas dextroxylohexosaminic acid and its lactone gave an hydro-*d*-idosaccharic and anhydro-*l*-saccharic acid respectively, the epichitosaminic acid and its lactone formed the same anhydro-*d*-mannonic acid. Thus, while in the first instance a Walden inversion took place, in either the acid or the lactone, in the second, the inversion occurred in neither or in both. Similar irregularities were observed by Fischer and his coworkers in their study of the action of nitrous acid on amino-acids and their esters.

Whether the lactone and the corresponding hexosamine yield the same deamino-acid could not as yet be established. Epichondrosamine has been obtained synthetically, but the sugar was found to possess such peculiarities that up to the present the attempt to convert it either into the amino-acid or into the deaminized acid has failed. The preparation of larger quantities of the sugar are now in progress.

#### EXPERIMENTAL.

The deamination of the amino-acids was brought about in the same manner for all acids. 5 gm. of the acid were dissolved in 50 cc. of 7.5 per cent hydrochloric acid. To the solution 5 gm. of silver nitrite were added. The flask containing the mixture was immersed in an ice and alcohol cooling mixture. The bath was allowed to warm up gradually to room temperature. After 12 to 16 hours a new portion of 2 gm. of silver nitrite and .2 cc. of 10 per cent hydrochloric acid was added. The material

was then allowed to stand 4 to 6 hours at room temperature. The resulting solution was freed from silver by means of hydrogen sulfide, the final filtrate concentrated, under diminished pressure, to a small volume and further treated according to the aim of the individual experiment.

Lactones were deaminized in a similar manner, the variation consisting in that 5 gm. of the lactone hydrochloride were dissolved in 50 cc. of distilled water, to which about 1 cc. of 10 per cent hydrochloric acid was added.

#### *Deamination of Epichitosaminic Acid.*

The mother liquor after deamination was reduced by means of aluminum amalgam according to the method of Levene and Meyer,<sup>4</sup> and the resulting solution neutralized with calcium carbonate, and concentrated under diminished pressure to a small volume. The composition of the Ca salt was the following.

0.1020 gm. substance gave 0.1244 gm. CO<sub>2</sub>, 0.0482 gm. H<sub>2</sub>O, and 0.0134 gm. CaO.

	Calculated for (C <sub>6</sub> H <sub>9</sub> O <sub>6</sub> ) <sub>2</sub> Ca+2H <sub>2</sub> O:	Found:
C.....	33.49	33.26
H.....	5.12	5.07
Ca.....	9.30	9.38

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.71 \times 100}{1 \times 2} = +35.5^\circ$$

Under the same conditions the lactone gave a salt of the following composition.

0.1030 gm. substance gave 0.1282 gm. CO<sub>2</sub>, 0.0484 gm. H<sub>2</sub>O, and 0.0138 gm. CaO.

	Calculated for (C <sub>6</sub> H <sub>9</sub> O <sub>6</sub> ) <sub>2</sub> Ca+2H <sub>2</sub> O:	Found:
C.....	33.49	33.94
H.....	5.12	5.26
N.....	9.30	9.56

<sup>4</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 599.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.71 \times 100}{1 \times 2} = +35.5^\circ$$

On deamination of chitosaminic acid chitaric acid is formed.

*Deamination of Epichondrosaminic Acid.*

In this instance the dicarboxylic acid possesses more convenient properties, and hence the product of deamination was oxidized further by means of nitric acid. For this purpose the solution obtained on deamination of 6 gm. of the amino-acid was concentrated to 35 cc., and an equal volume of concentrated nitric acid was added, the solution heated over a flame until the evolution of red fumes, and then the heating maintained for 7 minutes. The reaction product was rapidly evaporated with constant stirring on a clock glass placed on a boiling water bath. The residue was redissolved in a solution of 5 cc. of water and 5 cc. of nitric acid and again evaporated to dryness. The product was then evaporated twice with water in order to remove the adhering nitric acid. Finally the aqueous solution was converted into the calcium salt. The yield was 3.5 gm. of the crude calcium salt. The crude material was purified by removing the calcium by means of oxalic acid and reconvertng the filtrate into the calcium salt. The dry substance had the following composition.

0.0988 gm. substance gave 0.1042 gm. CO<sub>2</sub>, 0.0302 gm. H<sub>2</sub>O, and 0.0222 gm. CaO.

	Calculated for (C <sub>6</sub> H <sub>8</sub> O <sub>8</sub> Ca):	Found:
C.....	29.03	28.76
H.....	3.22	3.44
CaO.....	22.58	22.47

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.45 \times 100}{1 \times 5} = -9.0^\circ$$

Under the same conditions of oxidation chondrosaminic acid yields the inactive anhydromucic acid.

*Deamination of d-Dextroxylohexosaminic Acid.*

In the earlier work of Levene and LaForge it was found that xylohexosaminic acid and its lactone gave rise to *d*-anhydroidosaccharic and *l*-anhydrosaccharic acid. Since this work was carried out on a material which consisted of a mixture of the two epimers, it became necessary to verify these earlier experiments.

10 gm. of pure *d*-dextroxylohexosaminic acid were converted into anhydroidosaccharic acid in the same manner as previously described by Levene and LaForge. This time the substance crystallized after the first evaporation. It was taken up in a minimum amount of water and several volumes of acetone were added. On standing, the substance crystallized. The yield of recrystallized substance was 4.8 gm. The composition of the substance was the following.

0.1128 gm. substance lost on weighing 0.0186 gm.

	Calculated for $C_6H_{12}O_7 + 2H_2O$ :	Found:
H <sub>2</sub> O.....	15.80	16.49

0.0942 gm. dry substance gave on combustion 0.1282 gm. CO<sub>2</sub> and 0.0362 gm. H<sub>2</sub>O.

	Calculated for $C_6H_{12}O_7$ :	Found:
C.....	37.50	37.11
H.....	4.20	4.30

The rotation of the substance was  $[\alpha]_D^{20} = \frac{-1.56 \times 100}{1 \times 2} = -78.0^\circ$ , or calculated for the dry substance =  $-93.4^\circ$ .

On oxidation of the lactone, the acid potassium salt of anhydrosaccharic acid was obtained.

*Deamination of d-Levoxylohexosaminic Acid.*

7 gm. of the acid were deaminized in the way described above. The reaction product was brought, by distillation under diminished pressure, to a volume of 30 cc. An equal volume of nitric acid was added. The solution was heated over a free flame until the beginning of the evolution of fumes; it was then gently heated for 7 minutes after which the solution was transferred to a clock glass, and evaporated on a water bath to dryness. The residue

was again dissolved in a solution consisting of 5 cc. of nitric acid and 5 cc. of water. The final product was converted into the acid potassium salt in the manner described by Levene and LaForge. The yield of the crude product was 3.05 gm. The salt was recrystallized three times and then had the following composition.

0.1000 gm. substance gave 0.0366  $K_2SO_4$ .

	Calculated for $C_6H_7O_7K + H_2O$ :	Found:
K.....	15.70	16.03

The optical rotation was

$$[\alpha]_D^{25} = \frac{-0.75 \times 100}{1 \times 2} = -37.50^\circ$$

# THE COLORIMETRIC DETERMINATION OF PHENOLS IN THE BLOOD.

BY STANLEY R. BENEDICT AND RUTH C. THEIS.

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(Received for publication, August 20, 1918.)

In 1915, Folin and Denis<sup>1</sup> described a method for the determination of free and conjugated phenols in the urine, using a phosphotungstic-phosphomolybdic reagent, which, in the presence of sodium carbonate, gives a color reaction with phenols. The formula for the color reagent was published in 1912,<sup>2</sup> at the same time as the uric acid reagent. Later, Folin and Denis<sup>3</sup> applied the method to phenols in the feces. Little has been done since in phenol determinations, except by Dubin, who published papers on the formation and elimination of phenols under normal and pathological conditions,<sup>4</sup> and on the effect of feeding inositol on the elimination of phenols.<sup>5</sup>

In connection with some work which we have been doing on the chemical analysis of blood in carcinomatous patients, we attempted to apply the color reaction to the determination of phenols in the blood. A method has finally been worked out, which we believe to be reasonably simple and accurate.

In brief, our method provides for the determination of (a) uric acid in one sample of the blood, and (b) of uric acid and phenols in a second sample of the same blood. The phenol content of the blood is then obtained by difference. At present this procedure seems preferable to one based upon any attempt to separate the uric acid from the phenol, and to determine the

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<sup>1</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.

<sup>2</sup> Folin and Denis, *J. Biol. Chem.*, 1912, xii, 239.

<sup>3</sup> Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 507.

<sup>4</sup> Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69.

<sup>5</sup> Dubin, *J. Biol. Chem.*, 1916-17, xxviii, 429.



latter separately. Since, according to our technique, uric acid gives only about one-third as much color as does an equal weight of phenol, the error involved in correcting for the uric acid present is, we believe, negligible.

Blood filtrates freed from protein and treated with phenol reagent and 20 per cent sodium carbonate give solutions of a muddy green color, which are hard to read in the colorimeter and not very stable or satisfactory to work with. The solutions precipitate out easily. Dubin<sup>4</sup> also reports this green color in working with urines.

The "stabilizing" action of sulfites upon the reduction of photographic plates by phenols is a matter of common knowledge. This fact led us to test the influence of sulfite upon the development of color by the phenol reagent. The effect is quite remarkable. The dirty green colorations change instantly to a clear and brilliant blue upon the addition of the sulfite. The sulfite is added after the carbonate, and an excess of carbonate is always used. The final solutions match the standards perfectly in the colorimeter, and are remarkably stable. The range of accuracy of the reaction depends on the particular sample of sodium tungstate used in the reagent. The standard solutions never precipitate out, and the unknown, only when a large excess of potassium oxalate has been added to the blood to prevent clotting. In that case they can easily be centrifuged and read. The maximum color is reached in 20 minutes, and the color is so stable that a standard may be used for determinations all day; in fact, some standards have been kept 5 days without altering. Some unknown solutions were left for several days, then read against a standard simultaneously prepared. The results were almost identical, as the following tabulation shows. The figures indicate mg. per 100 cc. of blood.

1 Day.	3 Days.	1 Day.	3 Days.	1 Day.	3 Days.
4.52	4.58	4.56	4.53	4.93	4.64
4.16	4.36	4.06	4.90	4.94	4.90

Uric acid gives a blue color with the phenol reagent, but the color is only about one-third as intense as the phenol. As each fresh reagent is prepared, a known uric acid solution should be

compared with the phenol standard to determine the percentage of color produced by uric acid. The uric acid should be determined on the same specimen of blood, according to Benedict,<sup>6</sup> calculated in terms of phenol, and subtracted from the total phenol color.

Folin and Denis<sup>3</sup> use as standard a strong solution of phenol in 0.1 N HCl, and dilute from that. Phenol cannot be weighed accurately, and must be titrated with iodine and sodium thiosulfate. The dilute solutions keep only for a few days.<sup>4</sup> Therefore, a phenol which can be weighed directly would be more desirable. Pure resorcinol gives 86 per cent of the color given by phenol. We use as a standard a solution of resorcinol (0.581 mg. of resorcinol in 5 cc.) in 0.1 N HCl, which corresponds in color to 0.5 mg. of phenol in 5 cc. A solution five times this strength in hydrochloric acid has been kept without altering for 4 months, and the dilution has been made from that each week.

The method as used by us is as follows: Precipitate the proteins from 5 cc. of defibrinated blood with five times the volume of boiling 0.01 N of acetic acid. Add an equal volume of boiling water and 10 cc. of aluminum cream and filter while hot, washing out the casserole with boiling water several times. Boil the filtrate to about one-half volume and precipitate again with aluminum cream, or boil to smaller volume and precipitate with colloidal iron. Boil this filtrate to about 10 cc. and transfer quantitatively to a graduated cylinder. To this add 8 cc. of phenol reagent (made by boiling under a reflux condenser for 2 hours, 100 gm. of sodium tungstate, 20 gm. of phosphomolybdic acid, 50 cc. of 80 per cent phosphoric, and 750 cc. of water, cooled and diluted to 1 liter) and 3 cc. of 20 per cent sodium bisulfite. Mix immediately and make up with distilled water to from 50 to 100 cc., according to amount of color produced. Compare in a colorimeter after 20 minutes against a simultaneously prepared standard of resorcinol (0.581 mg. in 5 cc.) similarly treated and made up to 100 cc. The colorimeter is conveniently set at 20 mm. Calculate in terms of mg. per 100 cc. of blood.

Determine uric acid on the same specimen and calculate in terms of phenol. Subtract this from the total color with phenol

<sup>6</sup> Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

reagent, and the result represents mg. of phenol per 100 cc. of blood.

We have added known quantities of resorcinol to blood samples and have recovered about 85 per cent by the above method. Phenol itself added to blood disappears completely during the boiling of the blood filtrates. We have, however, assured ourselves of the practical absence of volatile phenols from blood by testing the distillate from the blood filtrates with the phenol reagent, with a negative result.

## PHENOLS AND PHENOL DERIVATIVES IN HUMAN BLOOD IN SOME PATHOLOGICAL CONDITIONS.

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(Received for publication, August 20, 1918.)

Phenols in urine have recently been determined colorimetrically by Folin and Denis<sup>1</sup> and by Dubin.<sup>2</sup> The Folin-Denis method has been adapted by the present authors to the determination of phenols in blood. The method as described in the preceding paper depends upon the blue color produced by the reaction of phenols with a phosphotungstic-phosphomolybdic reagent of Folin and Denis<sup>3</sup> in the presence of sodium carbonate and sodium sulfite. Uric acid, the chief other constituent of blood, giving a blue color with this reagent, is separately determined, and its value subtracted from the total.

Phenols have been determined in 83 specimens of blood, covering a variety of conditions. Cases 1 to 30 (Table I) were obtained from the Roosevelt Hospital through the courtesy of Dr. W. G. Lyle; Cases 31 to 83 from the Memorial Hospital through the cooperation of Dr. William S. Stone. The Roosevelt cases average 4.73 mg. per 100 cc. of blood, with a variation of 2.38 to 7.33 mg. Although thirteen different conditions are represented, little difference in the phenol value can be detected and that value bears no relation to the amount of uric acid present. The three hernia cases average rather higher than the others, which perhaps is due to increased intestinal putrefaction. Polycythemia case (No. 30) had been treated with benzene for some time, but the phenol is not increased. Folin and Denis<sup>3</sup> found an

<sup>1</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.

<sup>2</sup> Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69.

<sup>3</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.

TABLE I.

Case No.	Diagnosis.	Uric acid.	Phenol.
		mg. per 100 cc.	mg. per 100 cc.
1	Nephritis.	5.02	3.44
2	"	2.45	4.72
3	"	2.09	5.91
4	"	5.05	5.00
5	"	7.05	4.84
6	"	2.88	4.40
7	"	4.32	4.06
8	"	2.74	4.29
9	"	3.88	2.38
10	"	6.05	5.42
11	Pneumonia.	1.98	4.70
12	"	1.29	5.11
13	"	<1	4.53
14	Diabetes.	1.91	4.01
15	"	4.85	4.40
16	Fractures.	1.52	6.55
17	" and tabes.	3.86	3.63
18	Fracture.	2.47	4.93
19	Hernia.	1.20	5.43
20	"	2.45	7.33
21	"	2.13	5.66
22	Varicocele.	<1	6.90
23	Dysentery.	2.53	3.58
24	Raynaud's disease.	6.15	5.60
25	Tabes.	5.35	5.81
26	Empyema.	4.20	5.75
27	Neurasthenia.	2.57	3.21
28	Rheumatism.	1.61	4.28
29	"	1.45	6.73
30	Polycythemia.	3.17	4.18
31	"	6.18	4.38
32	Leukemia and tabes.	1.53	5.65
33	Fibroid of uterus.	<1	4.75
34	" " "	1.91	5.55
35	" " "	2.98	3.36
36	" " "	3.97	5.16
37	Carcinoma of uterus.	3.52	3.17
38	" " "	<1	2.52
39	" " "	<1	4.13
40	" " " (postoperative).	<1	5.30
41	" " " "	<1	5.25

TABLE I—*Concluded.*

Case No.	Diagnosis.	Uric acid.	Phenol.
		mg. per 100 cc.	mg. per 100 cc.
42	Carcinoma of uterus.	<1	4.91
43	" " "	<1	6.25
44	" " "	4.65	2.91
45	" " "	1.62	4.48
46	" " "	2.26	3.30
47	" " "	3.52	2.80
48	" " "	2.31	4.73
49	" " "	2.12	4.80
50	" " "	1.42	4.40
51	" " ovary.	1	5.87
52	" " "	2.39	3.03
53	Endometritis.	3.80	1.95
54	"	2.30	3.74
55	Lymphosarcoma.	1.80	7.68
56	"	1.50	5.15
57	"	1.91	4.72
58	"	2.86	3.17
59	"	1	5.19
60	Fascial sarcoma.	2.06	4.88
61	" "	1.73	5.36
62	Sarcoma of thigh.	3.22	6.01
63	" " sternum.	1.97	4.28
64	" " leg; gangrene.	2.53	7.23
65	Carcinoma of tongue.	2.52	4.10
66	" " "	1.96	4.14
67	" " tonsil.	<1	7.96
68	" " neck.	2.40	4.78
69	Keloid of neck.	4.25	5.55
70	Carcinoma of parotid gland.	2.36	3.22
71	" " " "	1.33	1.87
72	Epithelioma of inferior maxilla.	2.22	5.08
73	Carcinoma of stomach.	2.04	3.73
74	" " breast.	1.55	3.88
75	" " "	2.36	4.68
76	" " "	2.29	7.19
77	" " chest wall.	3.50	4.15
78	Hodgkin's disease.	<1	5.57
79	Mediastinal tumor.	1.63	4.08
80	Neoplasm in lung.	2.87	4.54
81	Tubercular cervical adenitis.	2.22	3.88
82	Abscess of liver.	2.11	5.27
83	Chronic gastritis.	2.62	3.05

increase from 0.3 to 0.87 gm. in the phenols excreted in the urine in a leukemia case, which was being treated with benzene.

The Memorial Hospital cases are cancer or allied diseases. Their average is 4.68 mg., with a variation of 1.87 to 7.96 mg. The only observation to be made here is that the sarcoma patients (lympho- and others) have an average of 5.36 mg., which is higher than the others.

Herter and Wakeman<sup>4</sup> determined the power of various tissues to conjugate phenols and indole by what they called the "contact test." A definite amount of tissue was brought into contact with a known solution of phenol and indole, then distilled

TABLE II.

Case No.	Uric acid + phenol.	Uric acid + conjugated phenol.	Case No.	Uric acid + phenol.	Uric acid + conjugated phenol.
	mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.
1	5.25	4.56	13	5.73	5.40
2	5.36	6.00	14	4.65	4.94
3	4.71	5.04	15	6.83	6.84
4	3.74	3.40	16	8.10	7.96
5	4.05	4.56	17	6.47	6.45
6	5.30	5.66			
7	5.93	4.94			
8	4.45	3.92			
9	5.49	4.84			
10	7.40	8.28			
11	4.15	4.33			
12	3.91	4.65			

and the distillate tested for phenol by Millon's reaction, and for indole, by the nitrosoindole test. By this method they found that the liver conjugated the largest amount of phenol and the brain and blood the least. In the urine Folin and Denis<sup>1</sup> found that the free phenols represented from 30 to 90 per cent of the total. We tested for conjugated phenols in the blood by treating 5 cc. as for the phenols, but to the final filtrate, in a test-tube we added eight drops of concentrated hydrochloric acid. This was heated to boiling, and then left in a boiling water bath for 10 minutes. The hydrolyzed solution was transferred to a graduated cylinder and the amount of phenol estimated colorimetrically.

<sup>4</sup> Herter, C. A., and Wakeman, A. J., *J. Exp. Med.*, 1899, iv, 307.

The results of Table II show that there are no conjugated phenols in blood, because while there is some variation, it is no greater than the error possible in the method.

By determining (a) uric acid, (b) total color with the phenol reagent, and (c) total color with the uric acid reagent, which reacts only with uric acid and polyphenols, it is possible to determine the relative proportion of mono- and of polyphenols in the blood. Work on twenty-two cases seems to indicate that the polyphenols represent about one-third of the total phenols. More work is being done in these determinations.

#### SUMMARY.

Phenols determined in a number of pathological cases show variations between 1.87 and 7.96 and average 4.70 mg. per 100 cc. of blood. The three hernia cases and ten of sarcoma average higher than the others.

The blood contains no conjugated phenols.

The polyphenols appear to represent about one-third of the total phenols.





## MUCINS AND MUCOIDS.

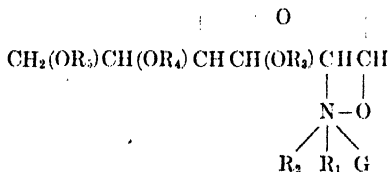
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(Received for publication, July 23, 1918.)

The structure of mucin and allied substances has remained in obscurity until recent years, although these substances interested both the chemist and the physiologist. Knowledge was lacking, particularly regarding the mode of union between the sugar and the rest of the molecule. The information given in text-books was generally limited to that of the solubilities and some other physical properties. The substances were classified in three groups: mucins, mucoids, and glucoproteins.

More recently Irvine and Hynd<sup>1</sup> had suggested the following structure of this group of substances.



According to this conception the amino group of the nitrogenous sugars serves as a bridge for the linking of amino-acids to the sugar, in a peptide as well as in a glycosidic union. The hypothesis suggested itself to Irvine and Hynd on the ground of their work on the glycosides of chitosamine, and was based entirely on speculative evidence. Irvine expressed the view rather tentatively, realizing the need of experimental proof.

On the other hand, evidence was accumulating tending to show that all substances allied to mucins contained in their molecule a complex acid which in its structure bore no similarity or relationship to peptides.

<sup>1</sup> Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1913, ciii. 41.

For many years it was known that an acid of the type of conjugated sulfuric acid existed in the molecule of chondromucoid.<sup>2</sup> Later its existence was revealed also in tendomucoid. The acid under the name of chondroitin sulfuric acid was discovered by Mörner, and received further attention through the efforts of Schmiedeberg at a time when methods applied to the study of carbohydrates were very imperfect. Later, in course of the work it was found that the acid could not be isolated with equal facility from every mucin or mucoid.<sup>3,4</sup> In many instances the removal of the protein radicle was accomplished only after numerous operations, and in course of these operations the conjugated sulfuric acid suffered partial decomposition. It was not clear (and it partly still remains so) whether the differences of individual mucins lay in the protein or in the acid radicles.

Finally a chemical distinction was discovered between the conjugated sulfuric acids of different origin.<sup>5</sup> The difference was in the components. Whereas one contained in its molecule the nitrogenous hexose, chondrosamine, the other had in its place chitosamine. For the former the name chondroitin sulfuric acid was retained, the latter was named mucoitin sulfuric acid.

The substances of the first group revealed a comparatively greater resistance towards hydrolytic agents and could be obtained in a fair degree of purity, whereas the substances of the second group when prepared free from protein always appeared in form of a mixture of mucoitin sulfuric acid with mucoitin. Also the intermediary substances showed a difference in their resistance towards hydrolytic agents, so that chondrosin could be prepared in a practically pure state whereas mucosin was found so labile that, under the conditions which permitted the preparation of the former, the latter was nearly completely hydrolyzed. On the other hand, when mucoitin sulfuric acid was subject to milder

<sup>2</sup> Mörner, C. T., *Skand. Arch. Physiol.*, 1889, i, 210.

<sup>3</sup> Levene, P. A., *Z. physiol. Chem.*, 1900-01, xxxi, 395.

<sup>4</sup> Mandel, J. A., and Levene, P. A., *Z. physiol. Chem.*, 1905, xlv, 386. López-Suárez, J., *Biochem. Z.*, 1913, lvi, 167. Alzona, F., *ibid.*, 1914, lxvi, 408.

<sup>5</sup> Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, 1916, xxv, 511; xxvi, 373.

hydrolysis a mucosin resulted which still contained undecomposed mucoitin.

Because of this instability of mucoitin sulfuric acid, the acid itself and the products of its partial hydrolysis were not obtained in the same degree of purity as chondroitin sulfuric acid and the corresponding products derived from it.

Furthermore, in course of the present work some differences were observed between various members of the mucoitin sulfuric acid group. It is true that, since the individual members were not always obtained in absolutely pure state, there is present also the possibility that these differences were brought about by impurities. However, the impression still remains that the acids of this group belong to two types. A representative of one type is the substance derived from funis mucin. The acid of this type is characterized by its gelatinous nature when precipitated by glacial acetic acid, by the comparatively small quantity of glacial acetic acid required for its precipitation, and by the readiness with which it forms an insoluble barium salt. The barium salt is very sparingly soluble in water, but is readily dissolved in the presence of acetates.

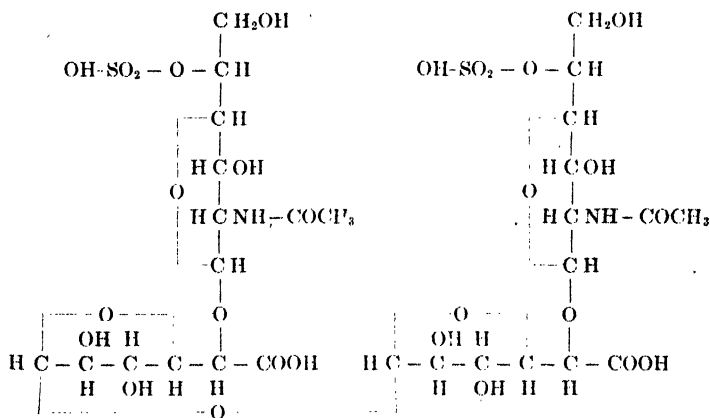
The second type of mucoitin sulfuric acid is characterized by the greater solubility of the barium salt, by the fact that the substance is precipitated by glacial acetic acid in form of a flocculent precipitate, and by the fact that a large excess of the acid is required in order to bring about the precipitation. A representative member of this type is the substance obtained from gastric mucus.

#### *Structure of Mucoitin Sulfuric Acid.*

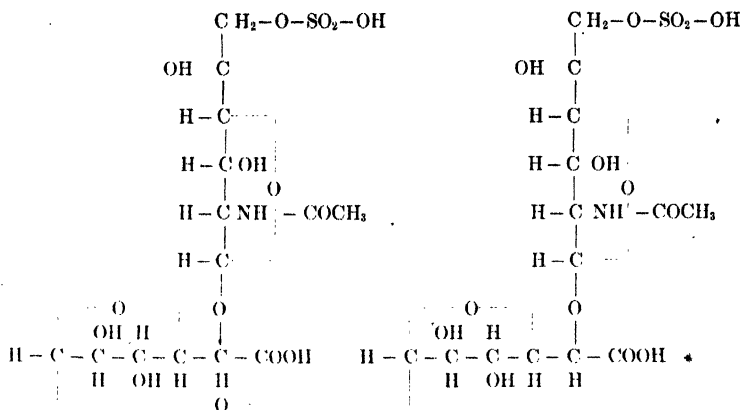
Concerning the structure of the acids of this group, it seems permissible to assume the same hypothesis as was previously formulated by Levene and La Forge for chondroitin sulfuric acid. On removal of sulfuric acid a substance is formed which is analogous to chondroitin. It is non-reducing, and does not contain free amino groups. On hydrolysis it forms mucosin, which is a disaccharide composed of glucuronic acid and chitosamine. Mucoitin, similarly to chondroitin, contains one acetyl group to each molecule of chitosamine, glucuronic, and sulfuric acids.

The glucuronic acid was identified in all the acids of the mucioitin sulfuric acid group but not with the same degree of exactness. Whereas in the acids of the funis mucin type the glucuronic acid was identified by the formation of furfural, by the analysis of the phenylhydrazine derivatives, and by the analysis of the acid potassium salt of saccharic acid formed on oxidation with nitric acid; in the acids of the second group it was demonstrated only by the furfural distillation, and by the formation of the phenylhydrazine, which, however, was obtained in a quantity too small for purification and analysis.

As regards the details of the structure all the arguments which suggested the structural formula of chondroitin sulfuric acid may be repeated for the suggestion of a graphic formula for mucioitin sulfuric acid. This is as follows:



On this occasion the structure of chondroitin sulfuric acid is presented for comparison though in a form slightly different from that originally suggested by Levene and La Forge. The difference is introduced in that part which expresses the structure of the amino sugar. In the early period of the work the aminohexose was assumed to be derived from *l*-ribose. It was later established that the configuration of the sugar was that of a *d*-lyxohexosamine. The substance is graphically presented as follows:



In both formulas the position of the sulfuric acid radicle, the place of union of sugar and glucuronic acid, and the position of the amino groups are arbitrary. To the sulfuric acid is assigned a different position in the two substances in order to indicate the existence of a difference in their respective behavior towards hydrolytic agents.

#### *Distribution of the Acids of Various Types.*

The distribution of the acids in various organs and tissues was found to be the following:

##### I. Chondroitin sulfuric acid.

- 1 Cartilage.
- 2 Tendons.
- 3 Aorta.
- 4 Sclera.

##### II. Mucoitin sulfuric acid.

- A.
  - 1 Funis mucin.
  - 2 Humor vitreous.
  - 3 Cornea.
- B.
  - 1 Mucin of gastric mucosa.
  - 2 Serum mucoid.
  - 3 Ovomucoid.
  - 4 Ovarian cysts.

Table I contains a summary of the analytical data obtained on the individual substances.

TABLE I.

	Theory.	Group I.				Group II.			
		Cartilage.	Tendons.	Aorta.	Sclera.	Navel cord.	Vitreous humor.	Cornea.	B.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	Gastric mucus. per cent Serum mucoid. per cent
$C_{26}H_{44}O_{23}N_2S_3Ba_2$ . Mucosin, chondroitin, sulfuric acid.	C	27.80	25.13		34.27	33.39	34.39	37.83	
	H	3.48	9.88		4.86	5.38	5.72	6.22	
	N	2.32	2.11	2.54	5.66	4.03	4.96	4.62	3.47
	S	5.30	3.72	2.40	4.57	4.07	3.63	3.10	1.48
	Basc.	22.70	18.35		28.83		21.50	14.74	29.14
Glucuronic acid derivatives.	Furfural.	33.0				17.5	23.0		13.0
	Acetyl.	10.0				9.39			7.8
	$[\alpha]_D$					-45.6			
$C_{12}H_{21}O_{11}N.HCl$ . Chondrosin or mucosin.			+	-	-	+			+
	C	36.9	37.3	35.8		38.25			
	H	5.64	5.66	6.0		5.8			
	N	3.58	3.45	3.45	4.34	-			
	NH <sub>2</sub> N	3.58	3.45	3.41	3.41	3.24			2.12
Sugar.	$[\alpha]_D$		+43.4	+41.5	+42.0	+25.55			+25.75
			Chondrosamine.				Glucosamine.		

## EXPERIMENTAL.

*Group I.**Aorta Mucoid.**Preparation of Chondroitin Sulfuric Acid.*

100 pounds of aorta freed from extraneous tissue were put through a hashing machine, taken up in 20 liters of a 2 per cent solution of sodium hydroxide, and allowed to stand for 36 hours. The extract was decanted and the residue again extracted for another 36 hours. The combined solutions were strained, neutralized, concentrated with an excess of barium carbonate, filtered, and finally precipitated with glacial acetic acid. The precipitate had the appearance of chondroitin sulfuric acid. The precipitate was washed with glacial acetic acid, then with alcohol, and dried. The yield was 40.0 gm. Of these 8 gm. were used for hydrolysis and the remaining material was purified in the following manner.

The material was again dissolved in water and precipitated with glacial acetic acid. The precipitate was washed with glacial acetic acid and then with alcohol. The dry precipitate was dissolved with the aid of potassium hydroxide. To this solution a slight excess over the required amount of barium chloride was added, followed by the addition of an equal volume of 95 per cent alcohol. The precipitate was washed by decantation with 50 per cent alcohol until free from barium chloride. The washing was then continued with alcohol of progressively increasing strength, finally with ether, and dried. The yield of this material was 25 gm.

*Analysis of Substance.*

0.200 gm. substance required for neutralization 3.63 cc. 0.1 N acid.

0.200 " " gave on fusion 0.0354 gm. BaSO<sub>4</sub>.

0.0778 " " " " combustion 0.0818 gm. CO<sub>2</sub> and 0.0386 gm.

H<sub>2</sub>O.

	Calculated for C <sub>22</sub> H <sub>44</sub> O <sub>17</sub> N <sub>2</sub> S <sub>2</sub> Baz:	Found:
C.....	27.8	28.7
H.....	3.48	3.35
N.....	2.32	2.54
S.....	5.30	2.4



*Hydrolysis of Substance for Identification of Sugar.*

8 gm. of the substance with 60 cc. of 20 per cent hydrochloric acid, together with 1.5 gm. of barium chloride and 1.5 gm. of stannous chloride, were hydrolyzed with reflux condenser for 12 hours. The product of hydrolysis was freed from barium and tin and the filtrate concentrated under diminished pressure, warmed to about 50°C. in a water bath. On concentration the sugar crystallized in long, microscopic, prismatic needles. These were transferred to a flask by means of alcohol containing hydrochloric acid. The flask was allowed to stand over night and the precipitate was then filtered and washed with alcohol and ether. The yield was about 1.0 gm. The melting point was 183° (uncorrected).

0.020 gm. gave in the Van Slyke apparatus 2.30 cc. of nitrogen gas at 25°C. and 762.7 mm. pressure.

	Calculated for $C_6H_{11}O_5N \cdot HCl$ :	Found:
N.....	6.51	6.40

The rotation of the substance was the following:

$$[\alpha]_D^{25} = \frac{\text{Initial.} \quad + 1.64 \times 2.0593}{1 \times 0.0508} = + 65.6^\circ \quad \text{Equilibrium.} \quad \frac{+ 2.27 \times 2.0593}{1 \times 0.0508} = + 91.15^\circ$$

*Preparation of Chondrosin.*—20 gm. of chondroitin sulfuric acid were hydrolyzed for 1 hour in a boiling water bath with 60 cc. of 20 per cent hydrochloric acid. The reaction product was filtered, concentrated under diminished pressure, and precipitated by means of alcohol and ether. The yield was 4 gm. The analysis of the substance gave the following results.

0.100 gm. required for neutralization 3.10 cc. 0.1 N acid.

0.020 gm. gave in the Van Slyke micro-apparatus 1.2 cc. N at 25°C., and 759.6 mm. pressure.

	Calculated for $C_6H_{11}O_5N \cdot HCl$ :	Found:
N.....	3.58	4.34
NH <sub>2</sub> -N.....	3.58	3.41

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{+1.04 \times 2.0026}{1 \times 0.0496} = +42.00^\circ$$

*Sclera Mucoid.*

*Preparation of Chondroitin Sulfuric Acid.*

For the preparation of this substance, originally the sclera and cornea were worked up in one. The hydrolysis of the substance, however, revealed the presence of two sugars, chitosamine and chondrosamine. Because of this in later experiments the cornea was dissected out. The sample used for ultimate analysis was prepared from sclera and cornea combined. But the sugar fraction still contained some glucosamine. On the other hand the mucoid from the cornea contained some glucosamine. The sclera was the only tissue which yielded mucoid containing both sugars, and the possibility is not excluded that one of them (chitosamine) is derived from adhering extraneous tissues.

The procedure for the preparation of the conjugated sulfuric acid was the following. The eyes were freed from adhering muscle and connective tissue, then the humor vitreous, lens, and retina were removed. Finally, the cornea and sclera were washed with running water from all extraneous material. After this the corneas were carefully dissected out, and the scleras were minced in the hashing machine, and placed in a large volume of a 3 per cent sodium hydroxide. For the corneas of 1,000 eyes 20 liters of sodium hydroxide were used. The extraction was continued 3 days, at the end of which time the solution was strained through cheese-cloth and neutralized with acetic acid. Barium carbonate was then added in excess and the mixture was concentrated on a water bath to a small volume. The product of the reaction was filtered on a suction funnel and then converted into the lead salt. The lead salt was treated in the usual way. It was converted into the sodium salt for the analysis of conjugated sulfuric acid. For the isolation of the sugar it was hydrolyzed directly.

*Analysis of the Sodium Salt.*

0.1903 gm. substance required for neutralization 7.69 cc. 0.1 N acid.

0.2855 " " gave 0.0950 gm. BaSO<sub>4</sub>.

0.0986 " " " 0.1239 gm. CO<sub>2</sub> and 0.0428 gm. H<sub>2</sub>O.

	Calculated for C <sub>20</sub> H <sub>44</sub> O <sub>10</sub> N <sub>2</sub> S <sub>2</sub> Ba <sub>2</sub> :	Found:
C.....	27.8	34.27
H.....	3.48	4.86
N.....	2.32	5.66
S.....	5.30	4.57
Ba.....	22.70	23.83

*Isolation of the Sugar.*—12.0 gm. of the lead salt with 2 gm. stannous chloride, 2 gm. of barium chloride, and 60 gm. of 20 per cent hydrochloric acid were heated with reflux condenser 10 hours over free flame. The reaction product was freed from lead, tin, and barium, and then concentrated to syrup. This was dissolved in a minimal amount of hot methyl alcohol. Soon crystals appeared which had the typical appearance of chitosamine. These were filtered off and the mother liquor allowed to stand, from time to time a few drops of ether were added. At the end of a week the maximum amount of chondrosamine hydrochloride settled out. The best yield for 1 kg. was 0.5 gm. of the sugar.

The melting point of this was 182° (corrected). The analysis of the substance was as follows:

0.020 gm. substance gave 2.30 cc. of nitrogen gas at 19°C. and 745 mm. pressure.

0.0523 gm. substance required for titration of the hydrochloric acid 2.39 cc. of AgNO<sub>3</sub> (1 cc. = 0.003546 gm.).

0.1036 gm. substance gave 0.1224 gm. CO<sub>2</sub> and 0.0602 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>13</sub> O <sub>5</sub> NHCl:	Found:
C.....	33.40	33.18
H.....	6.54	6.90
N.....	6.57	6.38
Cl.....	16.45	16.20

The rotation of the substance was the following:

$$[\alpha]_D^{25} = \frac{\text{Initial.}}{1 \times 0.0510} = +56.80^\circ \quad + \frac{\text{Equilibrium.}}{1 \times 0.0510} = +95.66^\circ$$

$$[\alpha]_D^{25} = \frac{+1.42 \times 2.0408}{1 \times 0.0510} = +56.80^\circ \quad + \frac{+2.39 \times 2.0408}{1 \times 0.0510} = +95.66^\circ$$

*Group II A.**Funis Mucin.**Preparation of Mucoitin Sulfuric Acid.*

Larger quantities of the material were accessible this year. This fact permitted more careful purification. It was observed in the course of preparation of this substance that to obtain it could not be accomplished satisfactorily by means of lead acetate. It is essential to free the cords from all adhering blood clots and blood vessels, as otherwise the resulting substance is contaminated with nucleic acid. The separation of the two is very troublesome. The purification, however, was accomplished by vigorous treatment with glacial acetic acid. The treatment with lead acetate is a convenient step in order to free the substance from adhering salt as well as from other impurities.

In removing the lead by means of hydrogen sulfide one has to bear in mind the insolubility of the acid in water. Because of this, it is necessary to carry out the separation of lead in a slightly alkaline solution.

The details of the process as carried out at present are as follows: About 100 cords, freed from blood vessels, either shredded or chopped in a hashing machine, are taken up in 6 liters of 72 per cent of NaOH and allowed to stand 3 days, then acidulated and centrifugalized to remove the precipitate. The supernatant liquid was concentrated with an excess of barium carbonate on a water bath. This operation was continued 24 hours and the product centrifugalized. The supernatant liquid was allowed to stand on a hot water bath after a second addition of barium carbonate. Water was added from time to time. The operation was continued about 2 days. The resulting material was then centrifugalized, and the supernatant liquid allowed to stand until part of the barium acetate had crystallized out. The material was again centrifugalized and the clear supernatant solution precipitated with glacial acetic acid. The precipitate was redissolved in water on addition of barium acetate; the substance was reprecipitated out of this solution with glacial acetic acid. The crude material was washed with 95 per cent alcohol to remove the excess of acetic acid. The material was dissolved in

water, and the solution was neutralized with a solution of barium hydroxide until it reacted neutral to litmus.

To the final solution enough 95 per cent alcohol was added to precipitate the crude barium salt. This was washed first with 50 per cent alcohol to remove adhering barium acetate, then with alcohol of increasing concentration and finally with 99.5 per cent alcohol and with ether. The final product analyzed as follows:

0.1000 gm. substance required for neutralization 2.88 0.1 N acid.  
 0.2000 " " gave on fusion 0.0592 gm. BaSO<sub>4</sub>.  
 0.0936 " " " " combustion 0.0450 gm. H<sub>2</sub>O and 0.1146 gm. CO<sub>2</sub>.

	Calculated for C <sub>22</sub> H <sub>44</sub> O <sub>10</sub> N <sub>2</sub> S <sub>2</sub> Ba <sub>2</sub> :	Found:
C.....	27.8	33.39
H.....	3.48	5.38
N.....	2.32	4.03
S.....	5.30	4.07

The optical rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{-0.50 \times 5.0641}{1 \times 0.0555} = -45.6^\circ$$

*Preparation of Mucosin.*—An attempt was made to prepare mucosin under the same conditions of hydrolysis as employed for preparation of chondrosin. However, the largest part of the substance underwent complete hydrolysis, with the formation of free chitosamine, which was identified in the usual way. It was found subsequently, that a substance analogous to chondrosin could be obtained under the following conditions.

4.5 gm. of the barium salt were dissolved in 100 cc. of 10 per cent hydrochloric acid and heated on a water bath for  $\frac{1}{2}$  hour. The solution then contained all its nitrogen in form of uncombined amino nitrogen, and showed a reduction of Fehling's solution equivalent to 1.12 gm. of glucose; the theory requires 1.26 gm. The solution was freed quantitatively from barium, concentrated to 3 cc. under diminished pressure at a temperature of water bath not exceeding 45°. These were then gradually poured into 200 cc. of alcohol, to which 400 cc. of dry ether had been added. A white flocculent precipitate then formed. It was allowed to stand over night, then filtered, and dried. The yield was 1.5 gm.

0.0188 gm. substance gave in the Van Slyke micro-apparatus 1 cc. N at 27° and 759.3 mm. pressure.

0.0918 gm. substance gave 0.1288 gm. CO<sub>2</sub> and 0.0476 gm. H<sub>2</sub>O.

	Calculated for C <sub>12</sub> H <sub>21</sub> O <sub>11</sub> N · HCl:	Found:
C.....	36.9	38.25
H.....	5.64	5.8
NH <sub>2</sub> N.....	3.58	3.24

$$[\alpha]_D^{20} = \frac{+0.66 \times 2.0287}{1 \times 0.0524} = +25.55^\circ$$

Later it was found possible to prepare mucosin by hydrolyzing the barium salt for  $\frac{1}{2}$  hour on a water bath in an aqueous solution of 1 per cent sulfuric acid. The excess of acid was then removed by means of barium hydroxide and the filtrate concentrated.

#### *Identification of Glucuronic Acid.*

The pressure of glucuronic acid was demonstrated by the formation of furfural on distillation of the mucoitin sulfuric acid with hydrochloric acid, by the phenylhydrazine derivative of glucuronic acid after the hydrolysis of mucosin with sodium amalgam, and finally by the isolation of the acid potassium salt of saccharic acid on oxidation of mucosin with nitric acid.

The estimation of the yield of phloroglucide of furfural also permitted an approximate estimate of the proportion of glucuronic in the molecule of the mucoitin sulfuric acid.

*Distillation with Hydrochloric Acid.*—1.5 gm. of barium salt were distilled over flame in 250 cc. of HCl (specific gravity 1.06) until the distillate no longer gave a test with alkaline acetate. To the distillate 0.3 gm. of phloroglucide was added and solution allowed to stand over night. The phloroglucine was filtered over a Gooch crucible. Yield was 0.0870 gm., which corresponds to 0.2610 gm. of glucuronic acid. The theory requires 0.5000 gm.

*Hydrolysis by Means of Sodium Amalgam.*—3.5 gm. of mucosin hydrochloride were dissolved in 50 cc., and 150 gm. of 2 per cent sodium amalgam were added in 25 gm. lots. After each addition the solution was neutralized with sulfuric acid. After the last portion of amalgam had been added the flask was placed in shaking machine for 5 hours and then allowed to stand over night.

The following day the solution was filtered and neutralized with sulfuric acid. 5 gm. of phenylhydrazine dissolved in 5 cc. of glacial acetic acid were then added and the solution was warmed on a boiling water bath for 30 minutes with reflux. On cooling over night a crystalline deposit formed. This was filtered, suspended in water, again filtered, and suspended in 99.5 per cent alcohol, filtered, and dried in a vacuum desiccator over sulfuric acid.

The melting point of the substance was 125°C. and decomposition with effervescence took place at 132°C. (corrected). A sample prepared from chondrosin had exactly the same melting point. In the communication of Levene and LaForge the melting point was given at 115°. The manner of purification of the substance as carried out at the later date was more rigorous, and the melting point of 125°C. with decomposition at 132° is to be regarded as the correct one.

0.0632 gm. substance gave on combustion 9.4 cc. nitrogen at 28°C. and 767.5 mm. pressure.

	Calculated for $C_{11}H_{12}O_4N_4 \cdot 1.5 H_2O$ :	Found:
N.....	17.17	16.92

*Oxidation with Nitric Acid.*—10 gm. of mucosin hydrochloride were dissolved in 10 cc. of distilled water to which 10 cc. of nitric acid (specific gravity 1.40) were added, and the solution was heated over free flame until the evolution of nitrous acid fumes became very lively. The solution was immediately transferred to a clock glass and evaporated with constant stirring. The subsequent treatment was as usual. The final solution was made up to 10 cc. Of these 1 cc. was used as control, and 9 cc. were allowed to digest with 2 cc. of a 50 per cent solution of potassium hydroxide on a boiling water bath for 2 hours. The solution was then made acid with acetic acid and the acid potassium salt was allowed to crystallize. The crude salt on fractionation out of water yielded a sample of the salt which analyzed as follows:

0.100 gm. salt gave 0.0356 gm.  $K_2SO_4$ .

	Calculated for $C_8H_7O_7K$ :	Found:
K.....	15.70	15.95

*Estimation and Identification of Acetic Acid.*

These experiments aimed to establish the number of acyl groups in the molecule, and to identify the character of the acyl groups.

*Quantitative Estimation of Acetic Acid.*—2 gm. of the barium salt of mucoitin sulfuric acid were dissolved in 200 cc. of water containing 15 gm. of barium hydroxide and hydrolyzed on a water bath for 5 hours. The product of the reaction was rendered acid to Congo red by means of sulfuric acid and filtered. The solution was then distilled guarding the original volume (600 cc.). The distillate was received in a measured volume of 0.1 N sodium hydroxide. 31.3 cc. of 0.1 N alkali were neutralized by the distillate. Calculated for acetic acid the yield was 0.1878 gm. The theory for one acetyl group requires 0.1944 gm.

*Identification of Acetic Acid.*—The entire distillate was concentrated under diminished pressure to 8 cc., rendered acid with sulfuric acid, and extracted with ether. To the ethereal extract a few drops of aqueous ammonia were added and the ether was allowed to evaporate spontaneously. The residue was converted into the silver salt.

0.1052 gm. dry substance gave 0.0681 gm. Ag.

	Calculated for $\text{C}_4\text{H}_5\text{O}_2\text{Ag}$ :	Found:
Ag.....	64.26	64.73

*Humor Vitreous Mucoid.**Preparation of Mucoitin Sulfuric Acid.*

To humor vitreous of 1,000 eyes enough of a 50 per cent sodium hydroxide solution was added to make the concentration of alkali 3 per cent. The material was allowed to stand 3 days; it was then acidulated and concentrated on a water bath after addition of an excess of barium carbonate. The final product was filtered on suction. To the filtrate enough of basic lead acetate solution was added to precipitate all of the acid. The crude lead salt was washed by decantation, then filtered and the precipitate was washed once with glacial acetic acid. The precipitate was then filtered and washed with alcohol. After this the substance was



taken up in water and the mixture rendered slightly alkaline by means of a solution of potassium hydroxide and the lead salt was decomposed by hydrogen sulfide. From the filtrate hydrogen sulfide was removed by aeration and the solution was poured into 2 liters of alcohol. A precipitate thus formed was washed with alcohol and ether. The substance analyzed as follows:

0.1000 gm. substance required for neutralization 3.54 cc. 0.1 N acid.  
 0.2000 " gave 0.0528 gm. BaSO<sub>4</sub>.  
 0.0958 " Ba salt of the substance gave 0.1208 gm. CO<sub>2</sub> and 0.0490 gm. H<sub>2</sub>O.

	Calculated for C <sub>25</sub> H <sub>44</sub> O <sub>22</sub> N <sub>2</sub> S <sub>2</sub> Ba <sub>2</sub> :	Found:
C.....	27.8	34.39
H.....	3.48	5.72
N.....	2.32	4.96
S.....	5.3	3.63
Base.....	22.7	21.5

*Nature of the Amino Hexose.*—6 gm. of the substance were dissolved in 30 cc. of 20 per cent HCl + 1 gm. of stannous chloride + 1 gm. of barium chloride heated with reflux condenser 8 hours over a Babo funnel. The solution was filtered, decomposed with hydrogen sulfide, and freed from barium quantitatively. The final solution was concentrated under diminished pressure to about 5 cc. The sugar began to crystallize in the distilling flask. The entire residue was taken up in methyl alcohol and allowed to crystallize at room temperature. The yield of the substance was 1.0 gm.

0.03018 gm. substance gave 3.47 cc. N at 19°C. and 745 mm. pressure.

	Calculated for C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> N · HCl:	Found:
N.....	6.51	6.45

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{1 \times 0.1010 \times 1.0018} = +96.77^\circ \quad \frac{\text{Equilibrium.}}{1 \times 0.1010 \times 1.0018} = +72.15^\circ$$

$$[\alpha]_D^{20} = \frac{+4.60 \times 2.1248}{1 \times 0.1010 \times 1.0018} = +96.77^\circ \quad \frac{3.43 \times 2.1248}{1 \times 0.1010 \times 1.0018} = +72.15^\circ$$

*Furfural Distillation.*—1.5 gm. of the barium salt were distilled with 250 cc. of HCl (1.06) so long as distillate showed the presence of furfural. Further treatment was carried out as

above, and 0.3 gm. of phloroglucine was added. The yield was 0.1165 gm., which corresponds to 0.3495 gm. of glucuronic acid. The theory required 0.500 gm.

*Cornea Mucoid.*

*Preparation of Mucoitin Sulfuric Acid.*

The corneas of 1,000 beef eyes were mechanically separated from the sclera and placed in 1,500 cc. of a 3 per cent sodium hydroxide solution and allowed to stand 3 days, then strained through cheese-cloth and acidulated with acetic acid. Barium carbonate was added in excess and all concentrated on a water bath to a thick syrupy mass containing the coagulated protein and the barium carbonate.

The mass was filtered on suction, and to the filtrate sufficient lead carbonate was added to precipitate all the acid. The purification of the lead salt was carried out in the manner described above. The final substance had the following composition.

0.1000 gm. substance required for neutralization 2.42 cc. 0.1 N acid.  
0.2000 " " gave 0.0330 gm. BaSO<sub>4</sub>.

A second precipitate was prepared as follows: The corneas were treated with alkali in the same manner as in the former experiment. The acidulated solution was concentrated in the presence of barium carbonate, and the filtrate poured into an excess of glacial acetic acid. The precipitate was washed repeatedly with glacial acetic acid, then with alcohol. The dry substance was then redissolved in a little water with the aid of potassium hydroxide. The solution was poured into a large excess of 99.5 per cent alcohol. The potassium salt obtained in this manner was dried and analyzed.

0.200 gm. substance required for neutralization 6.90 cc. 0.1 N acid.  
0.200 " " gave on fusion 0.0452 gm. BaSO<sub>4</sub>.  
0.0950 " potassium salt substance gave 0.1318 gm. CO<sub>2</sub> and 0.0528 gm. H<sub>2</sub>O.

	Calculated for C <sub>22</sub> H <sub>24</sub> O <sub>12</sub> N <sub>2</sub> S <sub>2</sub> Ba <sub>2</sub>	Found:	
		I	II
C.....	27.8		37.83
H.....	3.48		6.32
N.....	2.32	3.93	4.62
S.....	5.30	2.27	3.10
Base.....	22.70		14.74

*Hydrolysis of the Substance for Amino-Hexose.*—4.5 gm. of the substance were hydrolyzed in 60 cc. of 20 per cent hydrochloric acid, together with 1 gm. of barium chloride and 1 gm. of stannous chloride, and the solution was heated with reflux condenser for 12 hours over a Babo funnel. The solution as usual turned dark brown. It was diluted with an equal volume of water and was then freed from tin by means of hydrogen sulfide, and from barium by means of sulfuric acid. The solution was concentrated to a thick syrup. Glucosamine crystallized in the distilling flask. It was taken up in methyl alcohol and kept at room temperature in order to complete crystallization. The yield was 0.520 gm. The appearance of the crystals under the microscope was typical for glucosamine. The substance turned brown at about 200°C. and turned black at 220°C. It did not melt. 0.020 gm. of the substance gave in the Van Slyke apparatus 2.41 cc. of nitrogen gas at 27°C. and 756.8 mm. pressure.

	Calculated for $C_6H_{12}O_6N HCl$ :	Found:
N.....	6.51	6.80

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{1 \times 0.0485} = \frac{+2.27 \times 2.0390}{1 \times 0.0485} = +95.43^\circ \quad \frac{\text{Equilibrium.}}{1 \times 0.485} = \frac{+1.71 \times 2.0390}{1 \times 0.485} = +71.89^\circ$$

### Group II B.

#### *Mucin of the Gastric Mucosa.*

#### *Preparation of Mucoitin Sulfuric Acid.*

Mucus was removed from the gastric wall mechanically and a concentrated solution of barium hydroxide was added to make the total solution contain 3 per cent of the hydroxide. The solution was allowed to stand 3 days at room temperature. At the end of this time the solution was rendered acid to Congo red by means of sulfuric acid, then centrifugalized. The supernatant liquid was neutralized with a solution of barium hydroxide until neutral to Congo red, but still acid to litmus, and finally neutralized to litmus by means of barium carbonate, then boiled for about 3

hours, and filtered. To the filtrate again barium carbonate was added and the mixture was allowed to stand on a water bath from 2 to 3 days until a sample of the filtrate showed a negative biuret test. This was centrifugalized and the supernatant liquid precipitated by means of glacial acetic acid. The precipitate was redissolved in water and reprecipitated by means of glacial acetic acid. The precipitate thus formed was repeatedly washed with 95 per cent alcohol until most of the glacial acetic acid was removed. This material was then dissolved in a minimum amount of water, the solution was exactly neutralized with a solution of barium hydroxide, and the barium salt of mucoitin sulfuric acid precipitated by means of alcohol. The crude salt was repeatedly washed with a 50 per cent solution of alcohol until most of the inorganic impurities were removed, then with alcohol of progressively increasing concentration. This salt was a mixture of mucoitin sulfuric and nucleic acid. To separate the two the mixture was taken up in water and centrifugalized. The salt of the nucleic acid, being insoluble, was removed in this manner. To complete separation it was necessary to repeat the operation several times. Finally the clear solution was poured into an excess of alcohol giving a precipitate of the barium salt of the mucoitin sulfuric acid. A sample of the material prepared in this manner had the following composition.

0.1000 gm. substance required for neutralization 2.48 cc. 0.1 N acid.  
 0.1500 " " gave 0.0162 gm. BaSO<sub>4</sub>.

	Calculated for C <sub>28</sub> H <sub>44</sub> O <sub>10</sub> N <sub>5</sub> S <sub>2</sub> Bar:	Found:
N.....	2.32	3.47
S.....	5.30	1.48

$$[\alpha]_D^{20} = \frac{-0.19 \times 5.3035}{1 \times 0.0447} = -22.54^{\circ}$$

*Preparation of Mucosin.*—For the preparation of mucosin 14.0 gm. of the barium salt were dissolved in 100 cc. of water and 15 of concentrated hydrochloric acid, and allowed to stand on the boiling water bath 20 minutes. The solution was concentrated under diminished pressure (the temperature of the bath not exceeding 40°C.) to a volume of 5 cc. The solution was poured into a solution of 1 liter each of 99.5 per cent alcohol and ether.

A precipitate formed which was removed by filtration. The precipitate was then dissolved in about 3 cc. of water and precipitated by 400 cc. of 99.5 per cent alcohol. To filtrate, an equal volume of ether was added and thus a second precipitate was formed which reduced Fehling's solution, but was not yet free from mucosin.

0.010 gm. in the Van Slyke apparatus gave 0.38 cc. nitrogen at 22° and 753.7 mm. pressure.

	Calculated for $C_{12}H_{21}O_{11}NHCl$ :	Found:
NH <sub>2</sub> -N.....	3.58	2.12

The optical rotation of the substance was as follows:

$$[\alpha]_D^{20} = \frac{+0.23 \times 5.8780}{1 \times 0.0525} = +25.75^\circ$$

*Glucuronic Acid.*—The presence of glucuronic acid was demonstrated by furfural distillation. 1.0065 gm. were distilled with 250 cc. of hydrochloric acid (specific gravity 1.06). The yield of phloroglucine was 0.0440 gm. The theory requires 0.100 gm.

*Hydrolysis by Sodium Amalgam.*—7 gm. of mucosin prepared as above were dissolved in 100 cc. of water and 200 gm. of a 2 per cent amalgam were added in portions of 25 gm. at intervals. The entire operation lasted 24 hours. Before each new addition of amalgam the solution was neutralized with sulfuric acid. The final product was separated from mercury and filtered. To this solution were added 7 cc. of phenylhydrazine dissolved in 7 cc. of glacial acetic acid, and the entire solution was allowed to stand for 30 minutes with reflux on a boiling water bath. The reaction product was filtered from tar and allowed to stand at 0°C. over night. A small crystalline deposit formed. There was not sufficient material for purification or analysis.

*Acetyl Estimation.*—2 gm. of the barium salt were, with 300 cc. of water and 15 gm. of barium hydroxide, allowed to hydrolyze 5 hours, then neutralized, and the acetic acid was distilled into a 0.1 N solution of sodium hydroxide. The acid neutralized 26 cc. of the 0.1 N alkali, which corresponds to 0.156 gm. of acetic acid. The theory for one acetyl group requires 0.194 gm. The distillate was concentrated to very small volume. This was acidulated with

sulfuric acid and extracted with ether. From this the silver salt was obtained. It analyzed as follows:

0.1032 gm. substance gave 0.0662 gm. Ag.

	Calculated for $C_{21}H_{24}O_2Ag$ :	Found:
Ag.....	64.14	64.26

### *Serum Mucoid.*

#### *Preparation of Mucoitin Sulfuric Acid.*

The mucoitin sulfuric acid from this mucoid was prepared on one occasion by treatment of the entire serum, and on the other, by treatment of the protein obtained from the serum on coagulation by boiling.

The first sample was prepared in the following way. To 12.5 liters of the serum a 50 per cent solution of NaOH was added until the solution contained 3 per cent of alkali. It was allowed to stand 3 days at 40°C., then rendered acid by means of acetic acid, and concentrated on a water bath in presence of excess of  $BaCO_3$ . The filtrate was converted into the lead salt. This was treated with glacial acetic acid, dried with alcohol, freed from lead, and again reprecipitated with lead acetate. The lead salt was repeatedly washed in a mortar with glacial acetic acid. Finally it was washed with alcohol and dried. The yield of the dry substance was 14.0 gm. The substance contained 5.10 per cent nitrogen. This sample was used for hydrolysis.

The second sample was prepared from the coagulum obtained from 12.5 liters of beef serum. The process of preparation was exactly as in the above experiment. The lead salt was converted into the barium salt. The lead salt was suspended in water, and excess of barium carbonate was added, and hydrogen sulfide gas passed until all lead separated out. From the filtrate the hydrogen sulfide was removed by aeration. The solution was finally precipitated by means of 99.5 per cent alcohol. The precipitate was then dissolved in a little water and the mixture was centrifugalized to remove all insoluble Ba salts. This operation was repeated several times. Finally the solution was precipitated by means of 99.5 per cent alcohol. This substance was dried and yielded 1.5 gm. Analysis gave the following values.

0.1000 gm. substance required for neutralization 3.75 cc. 0.1 N acid.

0.1961 " " gave on fusion 0.0279 gm. BaSO<sub>4</sub>.

0.1008 " " " " combustion 0.1098 gm. CO<sub>2</sub> and 0.0400 gm. H<sub>2</sub>O.

0.1961 gm. substance treated with sulfuric acid gave 0.0971 gm. BaSO<sub>4</sub>.

	Calculated for C <sub>20</sub> H <sub>44</sub> O <sub>10</sub> N <sub>2</sub> S <sub>2</sub> Ba:	Found:
C.....	27.80	29.71
H.....	3.48	4.44
N.....	2.32	5.25
S.....	5.30	1.96
Ba.....	22.74	29.14

13 gm. of the first sample was taken up in 80 cc. of a 20 per cent solution of hydrochloric acid. 2 gm. of barium chloride and 2 gm. of stannous chloride were added. The solution was heated with reflux condenser 13 hours. The dark brown solution was filtered from melanin and diluted with an equal volume of water. The solution was freed from lead and tin by means of hydrogen sulfide and from barium by sulfuric acid.

The solution was concentrated under diminished pressure nearly to dryness. The residue was taken up in methyl alcohol and allowed to stand. Typical crystals of glucosamine hydrochloride separated out.

0.020 gm. in the Van Slyke apparatus gave 2.23 cc. nitrogen at 25°C. and 759.6 mm. pressure.

	Calculated for C <sub>4</sub> H <sub>13</sub> O <sub>2</sub> NHCl:	Found:
N.....	6.51	6.19

$$[\alpha]_D^{25} = \frac{\text{Initial.}}{1 + 0.0405} = \frac{+ 1.78 \times 2.0355}{1 + 0.0405} = + 89.46^\circ \quad [\alpha]_D^{25} = \frac{\text{Equilibrium.}}{1 \times 0.0405} = \frac{+ 1.42 \times 2.0355}{1 \times 0.0405} = + 71.37^\circ$$

The substance began to turn brown at 200°C. and turned black at 220°C. It did not melt.

# THE ANTISCORBUTIC PROPERTY OF DESICCATED AND COOKED VEGETABLES.

## AN EXPERIMENTAL STUDY.\*†

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(Received for publication, July 22, 1918.)

Drying or dehydrating vegetables is one of the many procedures advocated for preserving the available perishable food supply. The possibilities of conservation by desiccation are obvious. However, from a nutritional standpoint we know very little about the result of the use of dried foods. Drying on a large scale is a comparatively novel procedure in the preservation of vegetables for human consumption. Any innovation introduced in the preparation or preservation of foods ought to be supported by scientific evidence that it is physiologically justifiable. Accordingly, we have undertaken a study to ascertain what effect drying has on the antiscorbutic substance present in cabbage—a vegetable to which antiscorbutic properties have long been attributed.

It has been stated<sup>1</sup> that dried foods are valuable as an army ration, and the claim has been made that soldiers eat them without being able to tell by their taste whether the vegetables have been dried first. One of us has reported analyses<sup>2</sup> showing

\* This investigation was made possible by a grant from the Seessel Research Fund.

† A preliminary report of this work was made at the meeting of the Society for Experimental Biology and Medicine, May 15, 1918.

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<sup>1</sup> Murlin, J. R., *Science*, 1918, xlvii, 495.

<sup>2</sup> Givens, M. H., *J. Am. Med. Assn.*, 1918, lxx, 1743.



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the concentration in nitrogen and calcium in certain dried vegetables.

The antiscorbutic value of dried foods has been studied by other investigators. Holst and Frölich<sup>3</sup> found that potatoes, carrots, dandelions, and cabbage lost all or the greater part of their antiscorbutic property for guinea pigs through long drying. It appeared that the dried cabbage retained its prophylactic properties longer when kept in an incubator at 37°C. than in the open room. This result was attributed to the greater amount of moisture in the room. In contrast to the cabbage, the dandelion lost its antiscorbutic action immediately after drying. Chick and Hume<sup>4</sup> have reported that: "All the dried foodstuffs examined, including desiccated vegetables, were more or less deficient in the antiscorbutic vitamin." They do not state what dried vegetables were used. According to them, "The temperature at which the tissues are dried seems to be a matter of indifference."

Holst and Frölich found that fresh vegetables subjected to ordinary cooking still retained some of their antiscorbutic property, though often it was diminished to a slight extent. Cooking cabbage at 110–120°C. destroyed decidedly more of the antiscorbutic property than ordinary cooking.

To determine whether drying at various temperatures, or first cooking and then drying vegetables, destroys the antiscorbutic properties of these foods, we have conducted experiments on guinea pigs with cabbage and later with potatoes. If drying destroys entirely or reduces the antiscorbutic property of a vegetable, it ought to be revealed when that dried food is fed to a guinea pig which is receiving a diet known to produce scurvy. The results are summarized in Table I.

### *Drying of Cabbage.*

Cabbage dried at three different ranges of temperature has been employed. Lots 1 and 2 were dried at 42–52°C. ("low dried"); Lots 3, 4, and 5 at 38–43°C.; and Lots 6 and 7 at higher temperatures described later in detail ("high dried").

<sup>3</sup> Holst, A., and Frölich, T., *Z. Hyg.*, 1912, lxxii, 1; 1913, lxxv, 334.

<sup>4</sup> Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1917, x, 141.

In the preparation of the "low dried" cabbage the leaves were pulled apart, spread out on cheese-cloth in a wire basket which was elevated over a steam heated radiator. The dry air striking the cabbage on the bottom was of a temperature of 42–52°C., and the air at the top of the layer of cabbage 38–42°C. This procedure was continued for 2 to 4 days until all the cabbage was crisp. The water content of the various lots is shown in the tabulation below.

The "high dried" cabbage (Lots 6 and 7) was spread out in hot air ovens, regulated at 75°C. Of course, after placing the cabbage in the ovens the temperature dropped, in both cases to about 30°C. The temperature was then allowed to rise gradually. It had reached 70°C. in 8 to 10 hours, whereupon it was maintained for 2½ to 3 hours at 70–78°C. Then the cabbage was removed and placed on a wire tray over radiators at 65–70°C. and the drying completed in the course of the night. This cabbage differed from the "low dried" in that it was subjected to a higher temperature and to more moist heat. The heating in the oven darkened markedly some of the material, indicating caramelization of the sugar.

The "low dried" cabbage was kept in cotton gauze bags hanging in open rooms. It took up a slight amount of moisture in time as indicated by its slight loss in crispness. Unless otherwise noted this cabbage was fed as such in 1 gm. portions.

The "high dried" cabbage, ground, mixed in with the rest of the food in the proportion of 1 gm. per guinea pig per day, and the whole mixture dried as later described, was fed in this manner.

*Temperature of Drying and Water Content of the Samples.*

Lot.	Cabbage.			Temperature of drying. °C.
	Raw weight.	Dry weight.	Water content.	
	gm.	gm.	per cent	
1	2,855	235.0	91.8	43–53
2	1,650	139.1	91.6	42–52
3	3,618	271.3	92.5	38–43
4	3,287	279.0	91.5	38–43
5	3,505	348.5	90.1	38–43
6	2,980	261.2	91.2	65–73
7	2,630	232.6	91.2	65–78

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### *Composition and Preparation of the Diets.*

The diets contained soy bean flour, milk, yeast, paper pulp, sodium chloride, and calcium lactate. When "high dried" or cooked cabbage or cooked potato was used it was incorporated with the entire food mixture.

The soy flour was prepared for us<sup>5</sup> from unpressed and unheated soy beans. We heated the flour in an autoclave for 30 minutes at 20 pounds pressure. This procedure was necessary because the animals refused to eat the raw flour quantitatively. The heating markedly improved the palatability of the material, so that the flour thus prepared was eaten greedily by the animals.

Whole milk of high quality, containing 4 to 4.25 per cent fat, was fed. Yeast from a brewery was dried on steam heated radiators. The paper pulp was prepared from a cheap grade of filter paper purified by boiling for several hours in caustic soda, washing, boiling several hours in dilute hydrochloric acid, and washing free of acid. The sodium chloride and calcium lactate were pure.

The cooked cabbage was prepared by boiling finely minced cabbage in water for 30 minutes. The water in which the cabbage was cooked was added with the cabbage to the food mixture. This cooked cabbage was fed to each guinea pig in an amount equivalent to 10 gm. of raw cabbage per day. The cooked potatoes were prepared by peeling, chopping finely, and boiling them in water for 30 minutes. The water in which the potatoes were cooked was always added along with the potatoes to the food mixture. The potatoes were fed in an amount equivalent to 5 gm. of raw potatoes per animal per day.

The food was prepared by intimately mixing all components and drying the entire mass for 2 days on radiators at 65-70°C. The dried cake thus received was weighed and the same definite proportionate amount each time allotted to each animal. When the "high dried" cabbage or cooked cabbage or cooked potatoes were used they were incorporated with the other food components and the mixture was dried on radiators as above.

<sup>5</sup> Our thanks are due Dr. J. H. Kellogg and Mr. M. F. Deming for the soy flour used in these experiments.

Judging by the water content of the cabbage and the potatoes, both were fed in all experiments in an amount corresponding to approximately 1 gm. of dry material per guinea pig per day.

*Proportions of Dried Solids of the Diet.*

	<i>per cent</i>
Soy flour.....	76.3
Milk solids.....	11.4
Yeast, dried.....	2.9
Paper.....	2.9
Calcium lactate.....	2.9
Sodium chloride.....	2.9
	<hr/>
	100.0

The actual intake per animal per day of the various components of the diet was approximately:

Soy flour.....	11.1 gm.
Milk, whole.....	14.0 cc.
Dried yeast.....	0.44 gm.
Paper.....	1.1 "
Calcium lactate.....	0.44 "
Sodium chloride.....	0.44 "

It is a valid criticism of the usual scurvy-producing diets that they are deficient in other qualities as well as lacking in an anti-scorbutic property. The special soy bean mixture<sup>6</sup> used in the following experiments contained protein adequate for maintenance and growth, a sufficient quantity of necessary inorganic salts, presumably liberal amounts of fat-soluble and water-soluble vitamins, and some "roughage" in the form of paper pulp to promote the ready elimination of the feces.

*Behavior of Rats on the Soy Bean Diets Used.*

McCollum and Pitz<sup>7</sup> have emphasized the fact that the diet which produces scurvy in the guinea pig may be adequate in all respects for the rat. To determine whether this was the case with our special soy bean diet alone (*i.e.* without the addition of dried vegetables), it was fed *ad libitum* to six rats for a

<sup>6</sup> Cohen, B., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 102.

<sup>7</sup> McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.

period of more than 12 weeks. During that time these animals gained in weight, substantially at the same rate as the average normal rats of Osborne and Mendel.<sup>8</sup>

One female in this series gave birth to two apparently normal litters of four and six respectively. These young, however, did not survive longer than 3 days. Circumstances did not permit us to make proper provision for the care of them, and we are, therefore, unable to say whether their failure to survive was directly due to the diet of the mother or to inadequate care. However, our results indicate that this special soy bean diet is adequate for maintenance and growth of rats for a period fully six times as long as that in which the guinea pig develops symptoms of nutritional failure in the form of scurvy.

#### *General Procedure and Methods.*

The animals were mainly young or half grown, but there were also a few full grown ones. They were observed for a week or more before being started on a diet and were in good health at the beginning of these experiments. Two animals were placed in each metal cage, which was cleaned daily; as facilities permitted they were shifted so as to have a cage for each one. The animals were weighed on alternate days or oftener when it was deemed necessary. Once a day weighed amounts of food were placed in the cages. Distilled water was supplied *ad libitum*. During the experiments the animals were examined regularly to note the first appearance of the specific symptoms of scurvy later referred to. They were always present in animals which at autopsy showed the lesions of scurvy.

Necropsies were performed as soon after death as possible. When an animal seemed to be dying, it was chloroformed and autopsied at once. Special attention was directed to the appearance and condition of the mouth and bones for the usually accepted signs of this disease in the guinea pig.<sup>9</sup> These signs were hemorrhage and fragility of the bones of the extremities as well as loosening of the teeth. We did not observe hemorrhages in

<sup>8</sup> Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, 1911.

<sup>9</sup> Jackson, L., and Moore, J. J., *J. Infect. Dis.*, 1916, xix, 478.

the gums, though they were sometimes hyperemic in appearance. Spontaneous fractures of the wrists or ankle joints were frequently encountered. In our scorbutic animals the hemorrhages were subperiosteal, intramuscular, and subcutaneous in their order of decreasing frequency. The viscera were often congested and sometimes hemorrhagic, but there was rarely any impaction of feces such as would be associated with constipation.

Animals which died but did not reveal the above picture at necropsy were not classed as scorbutic. It might also be added that such animals did not exhibit tenderness of the joints during life.

## RESULTS.

### *Control Experiments.*

*Group V* represents a control experiment to show the effect of feeding the soy bean mixture without the addition of vegetables in any form. The animals in this series were nearly all mature which accounts for the somewhat deferred appearance of the first symptoms of scurvy. The average time of onset for half grown animals is about 16 days, while in this group it was 21 days. A typical curve (Guinea Pig 122) of body weight representative of this series is shown in Chart 1. It will be seen that feeding the soy mixture alone results in early onset of symptoms of scurvy succeeded by a decline leading to death unless, as proven elsewhere, suitable changes in the dietary are adopted.

### *Experiments with Various Vegetables.*

*Group I* demonstrates the effect of a limited amount of raw cabbage (10 gm. daily) added to the soy bean diet. One animal in this series died of pneumonia on the 47th day. The rest continued in good health until the end of the experiment (81 days). One of the latter was anesthetized and revealed at autopsy no sign of scurvy. The curve of body weight of Animal 105, Chart I, is typical. This experiment demonstrates the antiscorbutic quality of raw cabbage. For the sake of completeness, we might also add that not only will raw cabbage, like orange juice, prevent scurvy of the guinea pig under these conditions but it will

TABLE I.

Group and diet.	No.	Weight.			Duration until		Diagnosis.	Remarks.
		Initial.	Maximum.	Final.	Symptoms appeared.	Death.		
		gm.	gm.	gm.	days	days		
I. Soy diet plus 10 gm. of raw cabbage daily.	101	456	466	446		81	Normal.	Gave birth to 1 young 7 days after experiment started; young died. Still living.
	102	388	642	453				Gave birth to 2 young 60 days after experiment started; young survived 3 days. Anesthetized and found normal.
	103	143	287	287				Still living.
	104	437	598	575				" "
	105	310	469	434				" " (See chart.)
	106	152	282	187		47	Pneumonia.	
II. Soy diet plus 1 gm. of "low dried" cab- bage daily.	107	335	387	207	27	40	Scurvy.	Beginning 28th day ate very little of cabbage.
	108	407	413	216	27	30	"	" 28th " refused to eat cabbage.
	109	144	288	188	35	39	"	" 33rd " increased "low dried" cabbage to 2 gm. daily.
							Cause of death.	
	110	515	537	250	33	59	Pneumonia.	Gave birth to 2 dead young 7 days after experiment started. Increased cabbage to 2 gm. "low dried" on 33rd day. Refused to eat cabbage from 54th day on.

II. Soy diet plus 1 gm. of "low dried" cabbage daily.	111	374	503	286	*	35	Scurvy. Death due to prolapsed rectum.	Gave birth to 2 dead fetuses 19 days after experiment started. "Low dried" cabbage increased to 2 gm. daily on 33rd day.
	112	348	512	365	32	78	Scurvy.	Increased "low dried" cabbage to 2 gm. daily on 33rd day. Failed to eat "low dried" cabbage from 68th day on. (See chart.)
III. Soy diet plus 1 gm. of "high dried" cabbage daily.	113	525	529	324	25	29	Scurvy.	Changed to 1 gm. "low dried" cabbage on 27th day.
	114	387	387	228	22	46	"	22nd day changed to 5 cc. of orange juice and 2 gm. of "high dried" cabbage daily. Orange juice stopped on 26th day. On 35th day changed to 1 gm. of "low dried" cabbage daily.
	115	422	445	245	22	24	"	Bloody stools 20th day. Changed to 2 gm. of "high dried" cabbage daily on 22nd day.
	116	409	415	249	19	35	"	On 21st day changed to 5 cc. of orange juice and 2 gm. of "high dried" cabbage daily. On 26th day orange juice stopped.
	117	355	366	267	14	28	"	See chart.
	118	135	181	122	14	20	"	On 17th day changed to 2 gm. of "high dried" cabbage daily. On 18th day added 5 cc. of orange juice daily.

\* Died before external symptoms appeared.



TABLE I—Continued.

Group and diet.	No.	Weight.			Duration until		Diagnosis.	Remarks.
		Initial.	Maximum.	Final.	Symptoms appeared.	Death.		
		gm.	gm.	gm.	days	days		
IV. Soy diet plus cooked cab- bage equiva- lent to 10 gm. of raw cabbage daily.	125	477	477	277	18	27	Scurvy.	Changed to double amount of cooked cabbage on 22nd day. (See chart.)
	126	457	484	270	22	28	"	"
	127	159	220	164	14	50	Cause of death unknown.	5 cc. of orange juice daily from 21st to 35th day. Off cooked cabbage and to 1 gm. of "low dried" cabbage daily from 35th day on. Refused to eat "low dried" cabbage from 45th day on.
	128	417	426	300	14	34	Scurvy.	Changed to double amount of cooked cabbage on 26th day.
	129	337	368	245	19	73	"	5 cc. of orange juice daily from 22nd to 35th day. Changed to 1 gm. of "low dried" cabbage daily from 35th day on. On 45th day began to refuse some of the "low dried" cabbage.
	130	244	259	193	14	21	"	Changed to double amount of cooked cabbage on the 19th day.
	119	612	612	410	0	23	?	
V. Soy diet alone.	120	587	607	385	26	30	Scurvy.	Changed to 1 gm. of "low dried" cabbage daily on the 27th day.

V. Soy diet alone.	121	410	410	228	22	28	Scurvy.	Changed to 5 cc. of orange juice on 22nd day. See chart.
	122	468	463	278	23	28	"	5 cc. of orange juice daily from 20th to 27th
	123	334	334	215	16	66	"	day. 1 gm. of "low dried" cabbage daily from 27th day on. Refused to eat cabbage from 61st day on.
	124	415	419	220	17	23	"	5 cc. of orange juice on 21st day.
VI. Soy diet until scurbic, then 1 gm. of "low dried" cab- bage daily to supple- ment.	131	470	512	385	0	19	Scurvy. Cause of death, in- tussuecep- tion.	Gave birth to 3 large fetuses on 12th day.
	132	375	391	250	18	27	Scurvy.	Changed to 1 gm. of "low dried" cabbage daily on 21st day.
	133	169	249	190	10	41	"	Changed to 1 gm. of "low dried" cabbage daily on 22nd day. (See chart.)
	134	505	505	328	19	29	Pneumonia. ?	Changed to 1 gm. of "low dried" cabbage daily on 22nd day.
	135	420	480	438	18	81	Scurvy (?)	Changed to 1 gm. of "low dried" cabbage daily on 22nd day. Anesthetized. (See chart.)
	136	252	266	185	14	30	" Tuberculo- sis. Pneu- monia.	Changed to 1 gm. of "low dried" cabbage daily on 20th day.

TABLE I—Concluded.

Group and diet.	No.	Weight.			Duration until		Diagnosis.	Remarks.
		Initial.	Maximum.	Final.	Symptoms appeared.	Death.		
VII. Soy diet until scurbutic, then 1 gm. of "high dried" cab- bage daily to supple- ment.	137	gm. 408	gm. 408	gm. 284	days 0	days 6	? ? Scurvy.	Changed to 1 gm. of "high dried" cabbage daily on 16th day; 2 gm. daily "high dried" cabbage on 17th day; 5 cc. of orange juice daily on 18th day.
	138	487	487	337	20	8		
	139	197	266	169	14	23		
	140	502	502	337	20	21	" Cause of death, in- anition. Scurvy. " Pneumonia.	See chart. Changed to 2 gm. of "high dried" cabbage and 5 cc. of orange juice daily on 18th day. Off orange juice on 22nd day. Changed to 1 gm. of "low dried" cabbage daily on 34th day.
	141	422	464	265	21	26		
	142	239	249	199	14	43		

VIII. Soy diet until scurbutic, then cooked cabbage equivalent to 10 gm. of raw cabbage daily to sup- plement.	143	543	543	420	0	5	Inanition. Pneumonia.	Changed to double amount of cooked cab- bage on 19th day.
	144	352	352	182	16	23	Scurvy.	Changed to cooked cabbage on 21st day.
	145	347	347	237	16	27	"	Changed to cooked cabbage on 21st day.
	146	387	387	204	17	34	"	On 27th day cooked cabbage supple- mented with 1 gm. of "low dried" cab- bage daily. Refused to eat "low dried" cabbage beginning 29th day.
	147	289	316	179	14	25	"	Changed to double amount of cooked cab- bage on 18th day. (See chart.)
	148	181	262	179	14	27	"	Changed to double amount of cooked cab- bage on 20th day.
IX. Soy diet plus cooked po- tatoes equiv- alent to 5 gm. of raw potato daily.	161	272	308	198	15	28	Scurvy.	See chart.
	162	341	352	203	17	25	"	
	163	337	347	224	19	27	"	
	164	347	360	340	15		Still alive.	Supplemented with 10 gm. of raw cabbage daily beginning 20th day.
	165	314	336	200	14	28	Scurvy.	Supplemented with 10 gm. of raw cabbage daily beginning 20th day. Refused to eat last few days of life.
	166	313	340	333	17		Still alive.	Supplemented with 10 gm. of raw cabbage daily beginning 20th day.

also initiate a prompt recovery when added before irreparable damage has been done to the animal.

*Group III* is a series of experiments carried out to demonstrate whether or not cabbage dried at a comparatively high temperature still retains its antiscorbutic property. Our product did not delay nor prevent the onset of symptoms of scurvy, nor did it tend to ameliorate the scorbutic condition of the guinea pigs. For example, Animal 117, Chart I, is typical of the result of the use of "high dried" cabbage. If other measures were instituted in an attempt to save the animals, it is noted in the "remarks" column of the table.

*Group IV* represents a set of animals to which cabbage, subjected to ordinary cooking and then drying at a high temperature with the rest of the dietary components was fed. It will be seen from the table and the chart that this treatment of the vegetable yielded unfavorable results similar to drying the cabbage at a high temperature. Animal 125 is a typical example. Even feeding a double amount of the cooked cabbage was without any favorable effect.

*Group II* represents a series of experiments to determine whether a vegetable dried at a low temperature still retains its antiscorbutic property. The feeding of the "low dried" cabbage as a preventive measure delayed somewhat the appearance of symptoms in all the animals. The life of those guinea pigs, which ate the cabbage, was markedly lengthened and death correspondingly delayed. When the animals refused to eat the dried cabbage death soon intervened. Of course in the case of the two animals in which death occurred from other causes, no comparisons are permissible. The value of the "low dried" cabbage as a preventive is demonstrated by Guinea Pig 110, and by Guinea Pig 112, Chart I, which lived to 59 and 78 days respectively, death in the former being due to pneumonia and in the latter to scurvy because of a refusal to eat the cabbage.

*Group VII* clearly shows that the cabbage dried at a *high* temperature was without any favorable influence whatsoever. As a curative agent it apparently had no effect (Guinea Pig 141, Chart I).

*Group VIII* demonstrates that cabbage cooked and then dried is of no value as an antiscorbutic agent. The data do not war-

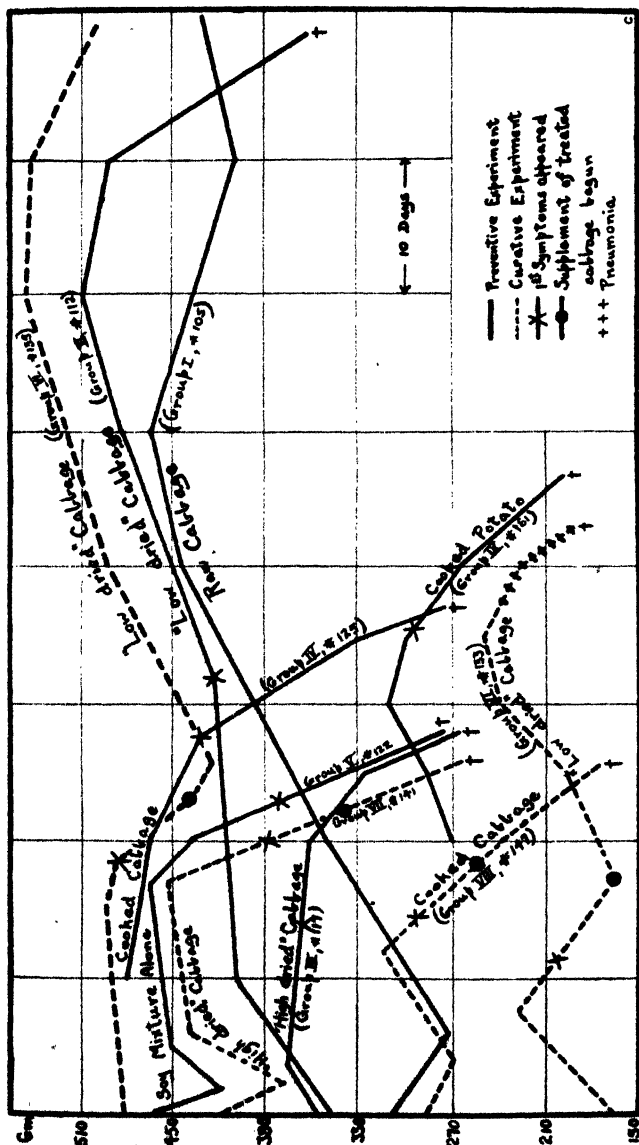


CHART 1. Typical curves of body weights in the various experiments.

The curve of Guinea Pig 135 (Group VI) is moved 60 gm. above actual weights to keep it from crossing the other curves.

rant any assumption relative to the influence of the cooking prior to the drying with the whole food mixture (Guinea Pig 147, Chart I).

*Group IX* is a series of experiments in which guinea pigs were fed potatoes first cooked and then dried with the rest of the food. This treatment of the food certainly destroyed any antiscorbutic property which the potato originally possessed. Whether there was not enough of the antiscorbutic substance present in the amount of the food fed or it was destroyed by the cooking or drying is still to be determined (Guinea Pig 161, Chart I).

*Group VI* demonstrates the effect of the "low dried" cabbage upon the course of the disease in animals already having distinct clinical symptoms of scurvy. In addition to the original six animals of this series we have records of eight other animals that were transferred from other diets as noted in Table I. At the end of the experiment (81 days), only one of the original six animals had survived. It was anesthetized and at autopsy only very slight lesions of scurvy were observed, indicating a recession of the marked scorbutic manifestations earlier in the experiment. The curve of body weight of this animal (Guinea Pig 135) is shown in the chart. Another animal followed a similar course, but succumbed to pneumonia. Autopsy in this case also revealed old receding lesions of scurvy but not fresh ones. The curve of this animal (Guinea Pig 133) is also shown in Chart I.

Several other animals in this series were allowed to decline too far before curative measures with "low dried" cabbage were adopted. These failed to respond and succumbed. The rest of them, *i.e.* those scorbutic animals that were treated with "low dried" cabbage in sufficient time, responded by maintaining themselves for comparatively long periods with some increase in weight until for some reason or other they refused to eat the cabbage, whereupon they promptly declined.

A critical review of the results here reported must take into consideration the experimental procedures employed. Two conditions of heating were concerned in the drying; in the case of the "low dried" material, dry air; in the case of the "high dried" and cooked, moist air. We cannot discuss the possible influence of aging, as all of our dried products were used in the

course of 30 days after their preparation. None of the containers in which the food was preserved was air tight. Consequently they took up a slight amount of moisture. Whether or not this exerted a deleterious effect, we can not state. Holst and Frölich are of the opinion that moist heat and preservation in moist air tend to destroy the antiscorbutic vitamine.

It is clear that the soy bean "mixture" alone as a diet will cause the appearance of scorbutic symptoms in the guinea pig within 2 or 3 weeks. We have also seen that supplementing this soy mixture with 10 gm. of raw cabbage daily effectively prevents the appearance of scurvy. This property of raw cabbage is effective, as a curative, precisely as is true of orange juice.

The antiscorbutic property is lost when the cabbage is cooked and dried in the manner we have indicated. In our experiments there is no sign of any effect on the appearance or course of scurvy by the addition of this cooked cabbage. A double amount was equally ineffective. As already stated, this cooked cabbage was subjected to a more severe treatment with moist heat than would ordinarily obtain, consequently it cannot be maintained that the mere cooking of cabbage destroys its antiscorbutic quality.

In the preparation of *dried* potatoes in any form for human consumption they are first subjected to cooking or steaming to destroy the enzymes which cause the material to darken. This exposes such material to moist heat more than pertains with other vegetables. Our experiments were planned to be comparable in that we first boiled the potatoes for 30 minutes and then dried them with the rest of the food at 65–70°C. We have not ascertained the potency in a quantitative way of potatoes but the amount fed was equivalent, in terms of dry solids, to the amount of cabbage used in the other experiments. The treatment imposed upon the potatoes in these experiments evidently destroyed any antiscorbutic substance in this food.

It is further obvious from our results that the drying of cabbage at a high temperature (70–80°C.) has the effect of destroying its antiscorbutic quality quite as thoroughly as did our cooking and drying. The "high dried" cabbage received a more moderate heat treatment than did the cooked; yet this lower tem-



perature range was sufficient to destroy effectually the antiscorbutic potency. Holst and Frölich have stated that this property is very susceptible to comparatively high temperatures, with moisture present,—a fact that the foregoing results readily confirm.

However, the "low dried" cabbage used in these experiments was not devoid of antiscorbutic value. Unless some other disease intervened or the animals refused to eat the cabbage, it delayed the onset of symptoms and prolonged life. Its action was not equivalent to that of the vegetable in the raw state. Hence the conclusion seems justified that *drying, even at a low temperature, diminishes the antiscorbutic property*. Nevertheless, the evidence clearly shows that the cabbage dried at the low temperature was not completely deprived of its antiscorbutic material while that dried at a high temperature was. These experiments confirm those of Holst and Frölich and of Chick and Hume that dried material may still contain a slight amount of antiscorbutic power. However, in so far as our experiments were conducted under more refined conditions they are more specific.

It has been stated repeatedly by McCollum and Pitz that unsatisfactory texture of the diet is the determining factor in scurvy of the guinea pig. Cohen and Mendel<sup>10</sup> using a variety of diets have shown that roughage as it affects the texture of a diet has no influence upon the course of scurvy; they conclude that the texture of a diet plays a secondary rôle, if any, in experimental scurvy. Our experiments substantiate this contention. Thus, equivalent amounts of cabbage were fed in the different groups. The only difference was in the heat treatment that the cabbage received. Yet scurvy could be cured or averted by the addition of 10 gm. daily of unheated cabbage, but there was no effect upon the symptoms when the same vegetable cooked or "high dried," or even twice the amount, was added. Therefore, it is difficult to understand how roughage could be the decisive factor in onset of the symptoms of the disease. The recent ex-

<sup>10</sup> Cohen, B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, May 15, 1918; *J. Biol. Chem.*, 1918, xxxv, 425.

periments of Hess and Unger<sup>11</sup> likewise cast doubt upon the rôle played by roughage in experimental scurvy of the guinea pig.

#### SUMMARY.

Experiments have been conducted to determine whether drying cabbage at a low temperature (38–52°C.), at a high temperature (65–78°C.), or cooking and drying at a high temperature (65–70°C.) destroys its antiscorbutic property. Potatoes, cooked and dried at a high temperature (65–70°C.), have also been studied.

A small daily addition of *raw cabbage* to a scurvy-producing diet was found to prevent scurvy in the guinea pig. Cabbage *dried in a blast of air at 40–52°C.* retained some of its antiscorbutic value in that it would considerably delay the onset of scorbutic symptoms, thereby prolonging life. Furthermore, it could be employed as a dietotherapeutic agent if the signs of scurvy were recognized early enough. The course of our experiments leads us to believe that the “low dried” cabbage will prevent scurvy in the guinea pig and initiate recovery from scorbutic symptoms, provided that the animal will consume a diet supplement of 1 gm. daily.

*Cabbage heated in an oven for 2 hours at 75–80°C., then dried at 65–70°C. for several days lost its antiscorbutic power. Cabbage cooked for 30 minutes, then subjected to drying for 2 days at 65–70°C. exhibited no potency as an antiscorbutic.*

*Potatoes cooked and dried for 2 days at 65–70°C., in the amounts here used, possessed no antiscorbutic value.*

These experiments also indicate that roughage is not the determining factor in the course of scurvy in guinea pigs. They also confirm the work of Cohen and Mendel in indicating that the antiscorbutic property is not identical with the so called fat- and water-soluble dietary essentials at present recognized.

We wish to acknowledge our thanks to Professor Lafayette B. Mendel for advice and criticism.

<sup>11</sup> Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 82.



# THE COLORIMETRIC ESTIMATION OF CHOLESTEROL IN BLOOD, WITH A NOTE ON THE ESTIMATION OF COPROSTEROL IN FECES.\*

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(Received for publication, August 31, 1918.)

During the past 6 years a number of different procedures have been utilized in the extraction of cholesterol from small quantities of blood for its ultimate colorimetric estimation. From the multiplicity of methods employed for the extraction process one would infer that they were not entirely satisfactory. Since 1913 a number of these methods have been tried out in our laboratory. In connection with a study of the blood lipoids in obesity, carried out in collaboration with Dr. Kast, it seemed necessary to investigate further the question of a suitable cholesterol method. For some time now, however, we have employed a comparatively simple and very satisfactory method,<sup>1</sup> which is described in the present paper.

Grigaut<sup>2</sup> was apparently the first to attempt the colorimetric estimation of cholesterol, using the Liebermann-Burchard reaction. 2 years later, Weston<sup>3</sup> made use of the Salkowski reaction for a similar purpose. Since these reactions are very delicate they at once afforded a means of estimating the cholesterol in small amounts of blood, thus furnishing an impetus to this type of

\*This study was made possible by funds contributed to the Laboratory through Professor Ludwig Kast of the Department of Medicine.

<sup>1</sup> A preliminary report of these observations was presented before the Society for Experimental Biology and Medicine, October 17, 1917; see Kast, L., Myers, V. C., and Wardell, E. L., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 1.

<sup>2</sup> Grigaut, A., *Compt. rend. Soc. biol.*, 1910, lxxviii, 791, 827; 1911, lxxi, 513.

<sup>3</sup> Weston, P. G., *J. Med. Research*, 1912, xxvi, 47. Weston, P. G., and Kent, G. H., *ibid.*, 531.

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investigation. Of the two reactions, the Liebermann-Burchard appears to have found somewhat greater favor, although both have been extensively employed. In addition to Grigaut and Weston, Autenrieth and Funk,<sup>4</sup> Henes,<sup>5</sup> Myers and Gorham,<sup>6</sup> Bloor,<sup>7</sup> Csonka,<sup>8</sup> Gettler and Baker,<sup>9</sup> Bernhard,<sup>10</sup> and others have described methods of cholesterol extraction in which these color reactions are used.

In the case of the excellent but laborious gravimetric digitonin method of Windaus,<sup>11</sup> for the estimation of total cholesterol, saponification of the cholesterol esters is necessary, since only the free cholesterol is precipitated by the digitonin. Cholesterol esters give the color reaction as well as does the free cholesterol. This fact does not appear to have been recognized until very recently, since the directions for the colorimetric estimation have almost invariably called for a preliminary saponification. As pointed out by Bloor, this saponification is unnecessary and the colorimetric estimation of the cholesterol thus becomes further simplified.

Bloor has suggested a method of extraction for the cholesterol which is very simple and would appear to be complete, but the results obtained with the method as finally carried out are higher than those by the older methods, and rather irregular, owing, apparently, to the presence in the extracts used of substances interfering with the Liebermann-Burchard color reaction for cholesterol. Other workers in this field, notably Mueller<sup>12</sup> and Weston,<sup>13</sup> have criticized the high results obtained with the Bloor method. They are of the opinion that these results are due in part to the admixture of brownish tints frequently obtained in the final development of the color. Luden<sup>14</sup> obtained similar high

<sup>4</sup> Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

<sup>5</sup> Henes, E., Jr., *Proc. N. Y. Path. Soc.*, 1913, xiii, 155.

<sup>6</sup> Myers, V. C., and Gorham, F. D., *Post-Graduate*, 1914, xxix, 938.  
Gorham and Myers, *Arch. Int. Med.*, 1917, xx, 599.

<sup>7</sup> Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

<sup>8</sup> Csonka, F. A., *J. Biol. Chem.*, 1916, xxiv, 431.

<sup>9</sup> Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.

<sup>10</sup> Bernhard, A., *J. Biol. Chem.*, 1918, xxxv, 15.

<sup>11</sup> Windaus, A., *Z. physiol. Chem.*, 1910, lxxv, 110.

<sup>12</sup> Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.

<sup>13</sup> Weston, J., *J. Biol. Chem.*, 1916-17, xxviii, 383.

<sup>14</sup> Luden, G., *J. Biol. Chem.*, 1917, xxix, 463.

results, but believed that these resulted from a combination of bile pigments and bile acids. Her data bearing on this point are very interesting. Our own observations regarding the Bloor method have likewise led us to the conclusion that its results are too high, and Baumann<sup>15</sup> has come to similar conclusions.

A description of the proposed method is given below, together with an adaptation to the estimation of coprosterol (?) in feces.

### *Methods.*

#### *Cholesterol Estimation in Blood.*<sup>16</sup>

1 cc. of blood, plasma or serum, is pipetted into a porcelain crucible or small beaker containing 4 to 5 gm. of plaster of Paris, stirred, and dried, preferably in a drying oven. It is now emptied into a small extraction shell (4 cm. long) and then inserted in a short test-tube (2.5 × 6 cm.), in the bottom of which are a number of small holes (Fig. 1).<sup>17</sup> This is now attached to a large cork on a small reflux condenser and the tube and cork are inserted in the neck of a 150 cc. extraction flask containing about 20 to 25 cc. of chloroform. Extraction is continued for 30 minutes on an electric hot plate, the chloroform made up to some suitable volume, such as 15 cc., filtered if necessary, and colorimetric estimation carried out as follows: 5 cc. of the chloroform extract are pipetted into a dry test-tube, and 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid (best with 0.1 cc. pipette) are added. After thorough mixing, the solution is placed in the dark for exactly 10 minutes<sup>18</sup> to allow the color to develop and then compared with a standardized aqueous solution of naphthol green B in a Duboseq or Kober colorimeter. The dye excellently matches the cholesterol color and appears to be permanent.

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<sup>15</sup> Baumann, L., Personal communication.

<sup>16</sup> In testing out various points in connection with the method we have been indebted to several individuals working in this laboratory during the past five years, Dr. F. D. Gorham, Mr. A. Bernhard, Dr. R. L. Kahn, and Dr. A. J. P. Pacini. The use of the dye was suggested to us by Mr. Bernhard, and Dr. Pacini made valuable suggestions in connection with the extraction.

The general problem of the cholesterol content of human blood has already been discussed, see Gorham and Myers, *Arch. Int. Med.*, 1917, xx, 599.

<sup>17</sup> We have generally used three extractors simultaneously on the same hot plate.

<sup>18</sup> In order to get the proper temperature for color development in warm weather it is advisable either to keep the reagents in a cool place or to insert the tubes in water during the development of the color.

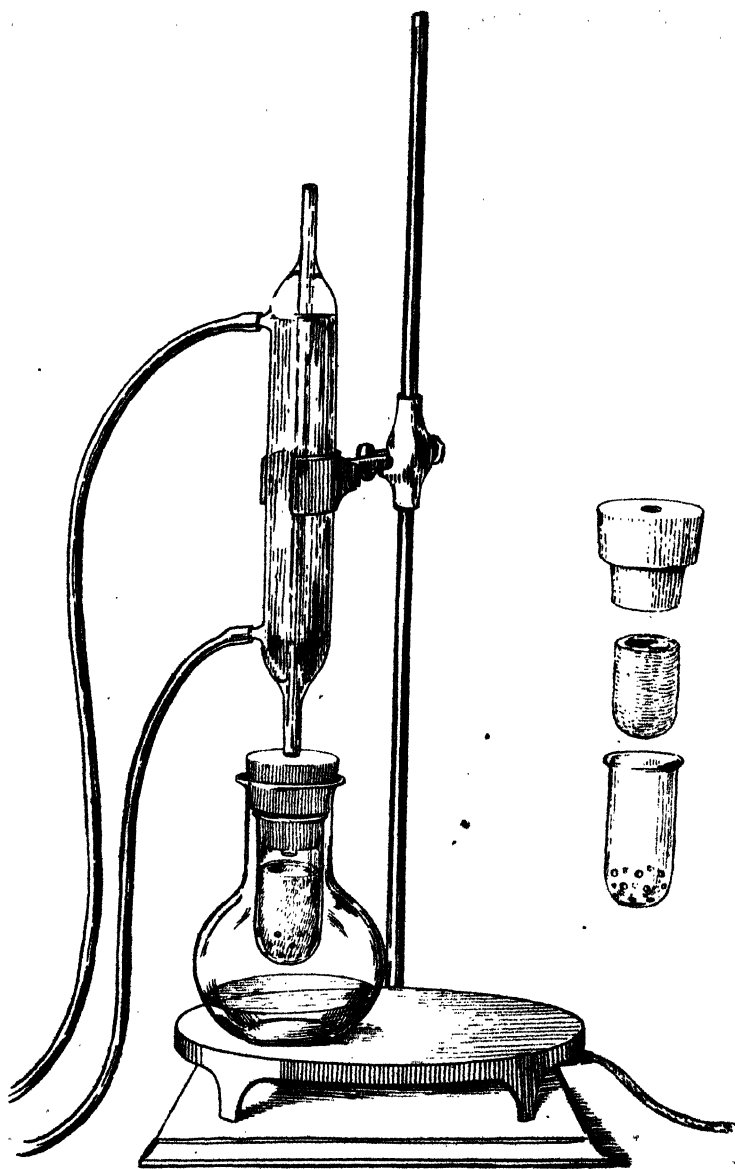


FIG. 1.

Regarding the use of the plaster of Paris, it would seem that, in addition to putting the blood into a finely divided and readily extractable condition, this calcium salt holds back substances which add to the color development with the Bloor technique. Chloroform appears to be a most excellent selective extractive here, and, in addition, is the solvent in which the color reaction must be carried out.

In conducting the extraction and developing the color it is important that the reagents should be perfectly anhydrous. The chloroform is best redistilled over calcium chloride while the acetic anhydride and sulfuric acid should be of known purity. On a number of occasions weak color development has been traced to acetic anhydride. For this reason we never develop a series of unknown solutions without first checking the quality of our reagents by developing a solution of pure cholesterol. With the method of extraction outlined above, brownish shades do not appear in the development of color. The use of the aqueous naphthol green B would appear to offer several advantages over the use of chloroform solutions of cholesterol as a standard. It appears to be rather more stable than the cholesterol in chloroform and does not evaporate as readily. With the dye it is not necessary to prepare continuously new standards to allow for the constant change in color.

Since the wedges and cups of the Hellige colorimeter are unaffected by chloroform, our first analyses were made with this instrument, though more recently the Duboscq has been used, mounting the cup employed for the chloroform in plaster of Paris, as suggested by Bloor. Still more recently we have had the clear glass cylinders of our Duboscq colorimeter replaced with black glass by the Klett Manufacturing Co. This removes the necessity for a light shield and renders the colors more easily matched. The present Kober instrument, in addition to the black cylinders has its clear glass bottoms fused on, a great advantage when using chloroform.

With our present lot of acetic anhydride, it has been found that when an 0.005 per cent solution of naphthol green B is used as a standard and set at 15.5 mm. on the Duboscq or Kober instrument, 0.4 mg. of cholesterol in 5 cc. of chloroform treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric



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acid will read 15 mm. The color curve for both the cholesterol and naphthol green B appears to fall in a straight line so that readings somewhat above or below the standard are accurate.

### *Coprosterol (?) Estimation in Feces.*

To 2 or 3 gm. of well mixed moist feces, accurately weighed in a porcelain casserole, are added 1 gm. of calcium hydroxide which is thoroughly mixed in with the feces. To this is then added 10 cc. of a 20 per cent solution of sodium hydroxide, and well stirred with a small glass rod. The casserole is now placed on the water bath and heated for about 2 hours with frequent stirring. When the mixture has evaporated almost to dryness, it is removed from the water bath, 3 to 4 gm. of finely powdered plaster of Paris are added, the mass thoroughly mixed, and dried in an oven at a temperature of 95° for 2 hours. The extraction and subsequent colorimetric estimation are carried out exactly as for cholesterol. A moisture determination should be made on another fraction of the same specimen so that the value of the coprosterol can be calculated in terms of percentage of the dry specimen. The calcium hydroxide is used to hold back any bile pigments. Saponification would, likewise, appear to be unnecessary here on account of the high fat content of feces. As we have found, liquid petroleum, now so commonly used therapeutically, may introduce a very disturbing factor in the determination.

Inasmuch as we have not yet been able to obtain coprosterol in a state known to be perfectly pure, it has been thought best to record the values in terms of cholesterol. Employing the usual technique of isolating coprosterol, we have been able to obtain an unsaponified light amber residue, which showed the needles supposedly typical of coprosterol (but no cholesterol plates). So far we have not been successful in isolating the needles. The colorimetric value of this dried residue, which is ordinarily weighed up as coprosterol, was found to be about one-third that of cholesterol, but identical in appearance.

The coprosterol content of dry feces, calculated as cholesterol, has been found to vary from 0.5 to 1.5 per cent. As yet we have not been able to note any correlation with the clinical findings in the cases.

We had planned a rather extensive study of the coprosterol content of the feces, but difficulties in obtaining entirely satisfactory quantitative specimens, as well as of ascertaining the exact color value for pure coprosterol have greatly delayed us. We have not,

however, given up hope of carrying out this work. Such a study would appear to be of considerable interest in certain of the obscure liver conditions, as well as in supposed disorders of cholesterol metabolism.

#### DISCUSSION.

As will be noted in the tables given below, this method fulfils three obviously necessary requirements. It gives consistent duplicates, completely recovers known amounts of added cholesterol, and satisfactorily checks with a recognized reliable method based on an entirely different principle (method of Windaus). From our data this does not seem to be true in the case of the Bloor method.

Table I presents a series of determinations with the method described above on normal human blood and blood to which either cholesterol or cholesterol palmitate was added. These were paralleled by similar determinations with the Bloor method. The duplicates and recoveries of added cholesterol would seem to be satisfactory with the method described, but this was not the case with the method of Bloor. It may be noted, however, that the data on the cholesterol palmitate with the proposed method bear out Bloor's contention that saponification of cholesterol esters is unnecessary for color development. With the Bloor method the cholesterol in alcohol-ether solution was added to the alcohol-ether to be used for the extraction, but with the proposed method a chloroform solution was added to the blood and plaster of Paris and the three were thoroughly mixed before drying. It may be stated that in the latter case the cholesterol was never intimately mixed with the blood, but we believe that such criticism is not valid.

Data are presented in Table II giving parallel determinations on the same samples with the Bloor method and the proposed method in comparison with the digitonin method. The checks of the proposed method with the Windaus gravimetric method are satisfactory, but this was not the case with the Bloor method.

Mueller<sup>12</sup> has made some very valuable suggestions regarding the Fraser and Gardner<sup>13</sup> technique of carrying out the digitonin

<sup>12</sup> Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 230.

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TABLE I.  
*Comparative Cholesterol Estimations.*

Bloor method.				Proposed method			
Colorimetric readings.	Cholesterol.	Cholesterol added.	Cholesterol recovered.	Colorimetric readings.	Cholesterol.	Cholesterol added.	Cholesterol recovered.
mm.	mg. per cc.	mg. per cc.	mg. per cc.	mm.	mg. per cc.	mg. per cc.	mg. per cc.
7.1	1.87			11.8	1.35		
7.6	1.76			11.9	1.34		
6.2	2.15			12.0	1.33		
6.2	2.15			11.9	1.34		
1 cc. of 0.08 per cent cholesterol solution per 1 cc. of blood.							
5.7	2.33	0.8	0.35	7.3	2.12	0.8	0.86
5.9	2.26	0.8	0.28	7.4	2.16	0.8	0.83
5.7	2.33	0.8	0.35				
6.1	2.18	0.8	0.20				
2 cc. of 0.08 per cent cholesterol solution per 1 cc. of blood.							
4.6	2.89	1.6	0.91	5.4	2.96	1.6	1.63
4.6	2.89	1.6	0.91	5.5	2.91	1.6	1.58
				5.3	3.00	1.6	1.67
4.6	2.89	1.6	0.91				
4.8	2.78	1.6	0.80				
1 cc. of 0.2 per cent cholesterol palmitate solution per 1 cc. of blood.							
4.9	2.72	1.24	0.74	6.2	2.58	1.24	1.25
4.8	2.77	1.24	0.79	6.0	2.56	1.24	1.23

At the time this series of observations was carried out our dye standard was set at 9.3 mm., which corresponded exactly with the color development of 0.4 mg. of cholesterol at 10 mm.

All observations in this series were on fractions of the same specimen of blood. Two estimations grouped together indicate the results of two colorimetric developments on the same extract.

determination, most of which we have followed. In view of the importance of this determination as a check on our own method, it may be well to outline the procedure as we have carried it out. It is as follows:

10 cc. of blood are saponified for 2 hours on the water bath with 100 cc. of 25 per cent aqueous potassium hydroxide. They are then shaken out first with 250 cc. of chloroform, and later with 125 cc., three times. The extracts are now combined and evaporated to dryness. The residue is dissolved in alcohol, and to the boiling alcoholic solution a 1 per cent solution of digitonin in 90 per cent alcohol is added, using about 25 per cent excess. This is allowed to stand over night in an ice box, and then filtered on tared hardened filter papers, washing first with ether and then with boiling water. The filter papers are placed in weighing bottles, dried in an oven, transferred to a desiccator for a short time, and weighed. The digitonin-cholesterol compound multiplied by the factor 0.2431 gives the cholesterol.

TABLE II.

*Comparative Cholesterol Values with the Digitonin, Bloor, and Proposed Methods.*

Blood.	Digitonin method.	Bloor method.	Proposed method.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1 Z.....	0.149	0.206	0.139
2 M.....	0.147	0.203	0.141
3 R.....	{ 0.125 0.125*	0.181	{ 0.124 0.130
4 Mixed blood.....	0.111		0.108
5 " ".....	0.158	0.187	0.164
6 Guinea pig.....	0.072	0.095	0.077
7.....		0.158	0.116
8.....		0.216	0.153
9.....		0.177	0.150
10.....		0.163	0.127
11.....		0.238	0.164

\* \* 20 cc. of blood used.

Mueller has pointed out that tared filter papers permit more rapid filtration than Gooch crucibles, and we can confirm him in this. As indicated by Fraser and Gardner, the use of ether and hot water is preferable for washing to the ether and alcohol used in the original method. Digitonin is not readily soluble in cold alcohol, but is easily soluble in boiling water and the excess of digitonin is thus easily removed. The digitonin-cholesterol compound is slightly soluble in alcohol, so the use of ether and boiling water obviates the slight loss in weight that might follow the use of alcohol for washing.

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### SUMMARY.

A method is described for the colorimetric estimation of cholesterol in blood, in which the cholesterol is directly extracted from the blood with the solvent (chloroform) employed in the development of the color reaction, thus rendering the estimation very simple.

Data are presented showing that good duplicates can be obtained with the method and added cholesterol completely recovered. Observations are likewise given in which the estimations excellently check those obtained with the Windaus gravimetric method.

Figures which we have obtained with the Bloor method are higher than those obtained with either the digitonin or the proposed method.

A modification of the method is described whereby it is possible to determine the coprosterol (?) of the feces.

# A NEW VOLUMETRIC METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD.

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(From the Robert Goelet Research Fund. Laboratory of Qualitative Analysis, College of the City of New York, with the Cooperation of W. G. Lyle, Harriman Research Laboratory, Roosevelt Hospital, New York.)

(Received for publication, August 9, 1918.)

## INTRODUCTION.

The object of this investigation was to devise a method for the rapid determination of uric acid in blood which would be free from the objections to the colorimetric method now universally employed. Aside from the general dislike which routine workers have for colorimetric methods, it has been found that different workers obtain on the same blood samples results which vary considerably. The gravimetric method, which depends upon the separation of uric acid as such from a cold solution strongly acid with hydrochloric acid, is altogether too time-consuming to be of service in clinical laboratories.

Morris<sup>1</sup> states that in a solution alkaline with sodium carbonate, uric acid may be completely precipitated as a zinc salt. This fact suggested a new method for the determination of uric acid in blood, the chief features of which would be the separation of the uric acid as a metallic salt and the estimation of the latter volumetrically by means of a standard solution of an oxidizing agent.

### *Experiments with Metallic Salts.*

It occurred to us that other metals besides zinc might be used as precipitants for uric acid and that among these some might be found which would be better than zinc. Accordingly we experimented with the following metals: zinc, tin, cadmium, aluminum, nickel, iron, cobalt, copper, and chromium.

<sup>1</sup> Morris, L., *J. Biol. Chem.*, 1916, xxv, 205.

The method was to add 10 cc. of a 10 per cent solution of the acetate of the metal to a solution of uric acid. In the experiments with Cu, Cr, and Fe, the chloride instead of the acetate was used. The metal was then precipitated as a carbonate and filtered off. To the filtrate were added 2 cc. of phosphotungstic acid<sup>2</sup> and 5 cc. of a 20 per cent sodium carbonate solution. The color obtained was then compared with standard colors. Controls were run with all experiments and the absence of uric acid was always confirmed by the addition of a small amount of this substance. Experiments showed that nickel and zinc are superior to the other metals; the latter either fail to carry down any uric acid or at best precipitate only a fraction of the amount present. Table I gives the results of one set of

TABLE I.

No.	Uric acid.	Metal.	Uric acid in filtrate.
	mg.		mg.
1	0.2	Zn	0.0
2	0.4	Zn	Trace.
3	0.2	Ni	0.0
4	0.4	Ni	0.0
5	0.2	Co	0.015
6	0.4	Co	0.02
7	0.2	Cd	0.05
8	0.4	Cd	0.1

experiments in which the efficiencies of Zn, Ni, Co, and Cd were compared. It is evident from Table I that nickel is slightly better than zinc as a precipitant for uric acid. Experiments with larger amounts of uric acid demonstrated more clearly the superiority of nickel. Sodium hydroxide, ammonia, and sodium phosphate were respectively tried in place of sodium carbonate. Experiments showed that sodium carbonate was the best.

Morris states that he could effect a separation of uric acid from phenol by precipitating the former as the zinc salt. Our experiments showed that a nickel salt may be used equally well for this purpose.

<sup>2</sup> Prepared according to the directions of Folin and Denis.

*Determination of Uric Acid in the Nickel Precipitate.*

The next step was to find a method for estimating the precipitated uric acid preferably by titration. A solution of potassium permanganate of normality 0.005 was tried but was found impracticable for the reason that the color of the nickel obscured the end-point and because a definite end-point could not be reached due to the rapid fading of the pink color. The removal of the nickel with hydrogen sulfide was not found practical owing to the difficulty experienced in boiling out all the hydrogen sulfide from the filtrate. We next tried a solution of iodine of normality 0.0004.<sup>3</sup> This was added in excess and the latter titrated with sodium thiosulfate solution of normality 0.0004<sup>3</sup> using starch as an indicator.

*Use of Glass-Stoppered Bottles.*—As will be shown later, it is necessary for the correct estimation of uric acid by iodine that the mixed solutions stand for half an hour. To determine whether or not any appreciable loss of iodine would result if the mixed solutions were allowed to stand in open beakers, the following experiments were made. 50 cc. of 0.0004 N iodine were added to beakers and glass-stoppered bottles each containing 50 cc. of distilled water and 10 cc. of nickel acetate.<sup>4</sup> After moistening the necks of the bottles with a little 10 per cent solution of potassium iodide, the bottles were tightly stoppered. At the expiration of 30 minutes, 4 cc. of starch solution were added to each of the beakers and bottles and the iodine in each was titrated with 0.0004 N thiosulfate. The results obtained are given in Table II.

The above results clearly show that in the beakers there was an appreciable loss of iodine. The necessity of employing glass-

<sup>3</sup> This was prepared by diluting 2 cc. of a stock 0.1 N solution to 500 cc. in a volumetric flask.

<sup>4</sup> Experiment showed that the so called pure nickel acetate contained an impurity which absorbed iodine. The following simple process was found efficient in removing this impurity. The nickel was precipitated with sodium carbonate and the precipitate quickly and completely separated by centrifuging. After pouring off the supernatant liquid, the precipitate was washed with water, the mixture centrifuged, and the clean liquid poured off. The precipitate was then dissolved in glacial acetic acid and the nickel was again precipitated, after which the precipitate was centrifuged, washed, and dissolved in acid.



stoppered bottles is therefore apparent. The figures above also supply some idea of the error of the titration which in this case is about 0.5 cc.

*Error of Titration.*—To determine more accurately the error involved in the final titration, the following experiments were made: To separate glass-stoppered bottles were added 10 cc. of 10 per cent nickel acetate, definite volumes of iodine solution delivered accurately from a burette, and sufficient water to bring the volume up to 60 cc. After adding 2 cc. of starch solution the iodine was titrated with 0.0004 N sodium thiosulfate solution. The results are given in Table III.

TABLE II.

50 cc. of 0.0004 N Iodine, 10 cc. of 10 Per Cent  $\text{Ni}(\text{C}_2\text{H}_3\text{O}_2)_2$ , 50 cc. of water.  
Time, 30 Min.

No.	Container used.	0.0004 N $\text{Na}_2\text{S}_2\text{O}_3$ used.
		cc.
1	Beaker.	41.9*
2	"	42.6
3	Glass-stoppered bottle.	50.0
4	" "	50.5
5	Bottle.	50.5 titrated immediately.

\* The end-point in these as in all other experiments was a green color which matched the standard color. This standard was prepared as follows: To a glass-stoppered bottle of the same size and type as that used for the titration, 10 cc. of 10 per cent nickel acetate are added. Water is then added until the volume approximately equals the final volume of the solution titrated.

Examination of the above figures shows that the largest error due to the titration is equivalent to 0.13 mg. of uric acid per 100 cc. The average error however is 0.065 mg.

Experiments were run to see if the error introduced by precipitating with zinc instead of nickel would be offset by a gain in accuracy in the titration due to the colorless end-point which the use of zinc would involve. It was found, however, that the green end-point gave a smaller error than that given by a colorless solution. Experiments were also made using different amounts of nickel solution. The results showed that with the volumes used in the method, 5 cc. of a 10 per cent solution was

the minimum amount. However, to insure an excess, 10 cc. were always used.

*Time of Reaction.*—It was observed that for the same amount of uric acid treated with iodine solution in glass-stoppered bottles, the iodine consumed varied with the time of standing; the longer the time the greater was the amount of iodine consumed. It was, therefore, important to ascertain the optimum time for the attainment of concordant results. To a series of glass-stoppered bottles were added 10 cc. of 10 per cent nickel acetate, a definite

TABLE III.

No.	Iodine.	Thiosulfate used.	Thiosulfate per 10 cc. of iodine.	Thiosulfate used, calculated from average.	Deviation.	Error in uric acid.*
	cc.	cc.	cc.	cc.	cc.	mg. per 100 cc.
1	10	8.82		9.23	0.41	0.08
2	10	8.98		9.23	0.25	0.05
3	20	18.00	9.00	18.46	0.46	0.09
4	20	18.20	9.10	18.46	0.26	0.05
5	30	27.80	9.27	27.69	0.11	0.02
6	30	27.78	9.26	27.69	0.09	0.02
7	40	37.60	9.40	36.92	0.68	0.13
8	40	37.36	9.34	36.92	0.44	0.08
Average.....			9.23†			0.065

\* The above values were calculated on the following basis: 1 cc. of iodine = 1 cc. of thiosulfate = 0.2 mg. of uric acid. These values while approximate were sufficiently accurate for this purpose.

† In calculating the average value for 1 cc. of thiosulfate in terms of iodine. Nos. 1 and 2 were not included since these determinations were subject to relatively large errors in consequence of the small amounts of standard solutions used.

volume of a standard solution of uric acid,<sup>5</sup> and finally 50 cc. of 0.0004 N iodine. The bottles were stoppered, allowed to stand for different periods of time, and then titrated. The results are given in Table IV.

Similar results were obtained in another series of experiments in which 0.8 mg. of uric acid were used. It appears from the figures in Table IV that the reaction is practically complete in

<sup>5</sup> This solution contained 0.4 mg. of uric acid per cc.

20 minutes, and that 30 minutes is a safe period for the reaction with amounts of uric acid such as are ordinarily present in 10 cc. of blood.

TABLE IV.

*0.4 Mg. of Uric Acid, 50 Cc. of 0.0004 N Iodine, 10 Cc. of 10 Per cent Ni(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>.*

No.	Time of standing.	Iodine consumed.
	<i>min.</i>	<i>cc.</i>
1	0.5	10.8
2	5	14.1
3	10	16.4
4	15	18.0
5	20	18.4
6	25	18.6
7	30	18.6
8	35	18.4
9	40	18.3
10	45	18.7
11	50	18.4
12	55	18.5
13	60	18.5

*Recovery of Uric Acid from Aqueous Solution.*

We next tested the efficiency of the method when applied to aqueous solutions of uric acid. The procedure was as follows: Definite amounts of a standard uric acid solution were measured into 50 cc. centrifuge tubes; 10 cc. of 10 per cent nickel acetate were then added and sufficient 20 per cent sodium carbonate solution to render the mixture alkaline.<sup>6</sup> The tubes were then centrifuged for 2 minutes at a speed of 3,000 R.P.M.<sup>7</sup> The supernatant liquid was poured off, and the precipitate washed by adding water and breaking up the cake with a glass stirring rod and stirring until an emulsion was formed. The tubes were then centrifuged again and the supernatant liquid was discarded. The precipitates in the tubes were treated each with 2 cc. of

<sup>6</sup> The mixture was thoroughly stirred during the addition of the sodium carbonate.

<sup>7</sup> The machine was capable of attaining a speed of 4,000 R.P.M. but the precipitate produced at this speed was caked too hard to wash thoroughly.

glacial acetic acid and the resulting clear solutions transferred to glass-stoppered bottles. By means of an accurate pipette 50 cc. of 0.0004 N iodine were introduced into each of the bottles and the latter stoppered after moistening the stoppers with a little 10 per cent potassium iodide solution. The bottles were well shaken, allowed to stand for 30 minutes, and titrated with 0.0004 N sodium thiosulfate. The results showed that practically all of the uric acid added was recovered.

It was found, however, in order to eliminate the blank given by sheep's blood, that a second precipitation of the uric acid with nickel was necessary. To determine how much uric acid could be recovered by a procedure involving a double precipitation of the uric acid, another set of experiments was made. The first precipitate obtained after centrifuging and washing was dissolved

TABLE V.

No.	Uric acid present.	Iodine consumed.	
		With no precipitation.	After two precipitations.
	mg.	cc.	cc.
1	0.2	10.07	10.20
2	0.4	20.80	20.05
3	0.6	30.49	30.22
4	0.8	40.60	40.97

in 2 cc. of acetic acid, carefully neutralized with 20 per cent  $\text{Na}_2\text{CO}_3$  solution and the latter added in slight excess. It was then centrifuged, washed, dissolved, and treated as above described. The results obtained in one series of experiments are given in Table V.

#### *Recovery of Uric Acid from Blood.*

10 cc. of sheep's blood were coagulated by boiling in 0.01 N acetic acid, treated with alumina cream, and filtered.<sup>8</sup> The concentrated serum was now treated by the procedure<sup>9</sup> already described for the recovery of uric acid from aqueous solutions, to

<sup>8</sup> For details of manipulation see description of method given later.

<sup>9</sup> In this preliminary work a single precipitation of the uric acid was made.

see if anything would be carried down in the nickel precipitate which would absorb iodine and hence give a blank. The results showed blanks equivalent to 0.4 to 0.6 mg. uric acid per 100 cc. of blood. Uric acid was added in different amounts to sheep's blood and the latter analyzed by the method described above.<sup>9</sup> Making allowance for the blank, the results showed that there was a loss in uric acid which was not proportional to the quantity of uric acid present. Definite amounts of uric acid were added to blood serum after coagulating the protein with acetic acid.<sup>8</sup> All the uric acid was recovered in addition to the blank. It was considered possible that the blank was due to oxalate used to prevent the clotting of the blood. Experiment however proved that this was not the case although the properties of oxalates show that they would be present during the iodine titration.

TABLE VI.

No.	Blood.	No. of precipitations.	Iodine consumed.	Equivalent in uric acid per 100 cc.
	cc.		cc.	mg.
1	10	1	3.65	0.73
2	10	1	2.98	0.60
3	10	2	-0.45	-0.08
4	10	2	0.40	0.08

Suspecting that the blank was due to protein carried down mechanically, it was hoped that by dissolving the precipitated nickel carbonate and uric acid and then reprecipitating, the blood blank might be eliminated. Experiments were first made with water solutions. The results have been given in Table V. The method of double precipitation was therefore applied to 10 cc. portions of sheep's blood with results shown in Table VI.

The figures in the last column clearly show that a double precipitation eliminates the blank which is invariably obtained in a single precipitation.

The method of double precipitation was now applied to sheep's blood to which definite amounts of uric acid were added. The results are given in Table VII.

The above results show considerable irregularity. In view of the fact that the recovery from water solution was fairly con-

stant, it would appear that the discordant results given above are due to errors entailed in the preliminary treatment of blood. This preliminary procedure, which is universally used in all methods, appears to us to lack definiteness so that not only different workers but the same individual will be unable to obtain concordant results. We are not aware of any published work in which this problem has been attacked; we have therefore made this topic our next object of study.

That the discordant results given in Table VII are due to the inadequacy of the present procedure for the coagulation and treatment of the blood, is confirmed by the results obtained in the following set of experiments. To separate 10 cc. portions of blood serum derived from an equal volume of blood, which had been coagulated in the usual manner, treated with alumina

TABLE VII.

No.	Quantity of blood used.	Uric acid added.	Uric acid recovered.	Amount recovered.
	cc.	mg.	mg.	per cent
1	10	0.2	0.164	82.0
2	10	0.4	0.358	89.5
3	10	0.6	0.526	87.6
4	10	1.0	0.935	93.5

cream, filtered, and concentrated, definite amounts of uric acid were added and the resulting clear solutions analyzed by our method. The results given in Table VIII were obtained. The excellent recovery of uric acid clearly points to the preliminary procedure as the cause of losses and irregularities of Table VII. The method proposed in this paper must be judged not by the results of Table VII but by those given in Table VIII.

Since the preliminary treatment is the same in the colorimetric and volumetric methods, it was thought worth while to run a series of experiments in which the two methods were compared. Sheep's blood, to which definite amounts of our standard uric acid solution were added, was analyzed by the colorimetric<sup>10</sup> and volumetric methods. The same standard solution of uric acid

<sup>10</sup> We are indebted to Miss Theis of the Memorial Hospital for making the above analyses.

was used as a basis in both methods. The results are given in Table IX.

The results show that the recovery of uric acid from blood is subject to considerable variation and that this is common to both methods. There seems to be little doubt that the irregularities and losses shown in Table IX are directly due to the inadequacy

TABLE VIII.

*10 Cc. of Serum Derived from 10 Cc. of Sheep's Blood.*

No.	Uric acid added.	Uric acid recovered.
	<i>mg.</i>	<i>mg.</i>
1	0.2	0.191
2	0.2	0.208
3	0.4	0.415
4	0.4	0.416
5	0.6	0.610
6	0.6	0.630
7	0.8	0.805
8	1.0	0.950
9	1.0	0.945

TABLE IX.

No.	Uric acid added.	Uric acid recovered by	
		Volumetric method.	Colorimetric method.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	0.333	0.314	0.234
2	0.333	0.261	—
3	0.460	0.378	0.382
4	0.570	0.391	0.420
5	0.667	0.558	0.460
6	0.667	0.607	—
7	0.700	0.552	0.585
8	0.700	0.547	0.565

of the preliminary treatment which is common to both methods. It is equally apparent that the volumetric method may be relied upon to give as good an indication of the uric acid content of blood as the colorimetric method. In Analyses 7 and 8, sufficient sheep's blood to which a known amount of uric acid had been added was coagulated, treated with alumina cream, filtered, and

concentrated in the usual manner. 10 cc. portions of this serum were then analyzed by both methods. It is of interest to note in these cases the agreement of the results by both methods, pointing unmistakably to losses entailed in the preliminary treatment of the blood.

#### *Method.*

Based on the work recorded above the following method was developed. 10 cc. of blood are run out from a pipette into 50 cc. of boiling 0.01 N acetic acid contained in an aluminum casserole. The mixture is stirred till the blood is coagulated as shown by the white foam which forms; 10 cc. of alumina cream are added and mixed by giving the casserole a rotary motion. The contents of the casserole are then filtered on a 18.5 cm. filter paper. The filter paper and coagulum are washed twice with 50 cc. portions of water heated to boiling in the casserole. The filtrate and washings are received in a porcelain casserole and boiled down to about 10 cc. 2 cc. of alumina cream are added and the mixture is filtered and washed twice with 10 cc. portions of boiling water. The filtrate and washings are received in a centrifuge tube of 50 cc. capacity. 10 cc. of 10 per cent nickel acetate solution are then added, thoroughly stirred, and 5 cc. of 20 per cent sodium carbonate solution added drop by drop with constant stirring. The tubes are centrifuged at about 3,000 R.P.M. for 2 minutes. After pouring off the supernatant liquid 40 cc. of water are added and the precipitate is thoroughly broken up with a stirring rod till an emulsion is formed. It is then centrifuged again. The supernatant liquid is poured off, 1 cc. of glacial acetic acid is added, and the mixture stirred till all is dissolved. 4 cc. of 20 per cent sodium carbonate are added to nearly neutralize the excess acid and then 6 cc. more are added drop by drop with constant stirring. After bringing up the volume to 40 cc., the mixture is centrifuged and washed as before. The precipitate is dissolved by adding 2 cc. of glacial acetic acid (cold) and the resulting solution transferred to glass-stoppered bottles of about 250 cc. capacity. The tube is rinsed twice with 5 cc. of water, and the rinsings are united with the solution. 50 cc. of 0.0004 N iodine are introduced into the bottle from a pipette, the stopper is moistened with a few drops of a 10



per cent solution of potassium iodide, the bottle tightly stoppered, shaken, and allowed to stand for 30 minutes. 2 cc. of starch solution are then added and the solution is titrated with 0.0004 N sodium thiosulfate to a green end-point.<sup>11</sup> The iodine and thiosulfate solutions are best kept as 0.1 N solutions. The dilute solutions are prepared as needed by diluting 2 cc. of the 0.1 N to 500 cc. in a volumetric flask.

#### *Standardizations and Calculations.*

After the dilute solutions of iodine and thiosulfate have been prepared as directed above, they are standardized as follows:

1. *Standardization of the Thiosulfate Solution.*—To 10 cc. of 10 per cent nickel acetate (free from impurities which consume iodine as shown by test) contained in a 250 cc. narrow mouthed glass-stoppered bottle add 50 cc. of iodine (0.0004 N) solution from a pipette and 2 cc. of starch solution, and immediately titrate with the thiosulfate solution (approximately 0.0004 N) till the color is matched by a control.<sup>12</sup> The latter is prepared by diluting in a similar bottle, 10 cc. of 10 per cent nickel acetate to approximately the final volume of the solution titrated. The number of cc. of iodine used divided by the number of cc. of sodium thiosulfate will give the value of 1 cc. of thiosulfate in terms of iodine. Thus 1 cc. of thiosulfate =  $x$  cc. of iodine.

2. *Standardization of the Iodine Solution in Terms of Uric Acid.*<sup>13</sup>—Introduce into separate 50 cc. centrifuge tubes definite quantities of the phosphate uric acid standard (0.4 and 0.6 mg. are suitable amounts). Add 10 cc. of 10 per cent nickel acetate, stir, and precipitate twice as directed above in the method. The

<sup>11</sup> The end-point in these as in all other experiments was a green color which matched the standard color. This standard was prepared as follows: To a glass-stoppered bottle of the same size and type as that used for the titration, 10 cc. of 10 per cent nickel acetate are added. Water is then added until the volume approximately equals the final volume of the solution titrated.

<sup>12</sup> A number of determinations should be made by one inexperienced with the method in order to familiarize himself with the end-point which, after a little practise, can be seen without much difficulty.

<sup>13</sup> This may be done simultaneously with a number of blood analyses, after the latter have been brought to the point when the uric acid and serum are contained in centrifuge tubes.

precipitate is dissolved in 2 cc. of glacial acetic acid and the solution transferred quantitatively to a glass-stoppered bottle. 50 cc. of the iodine solution are introduced by means of a pipette and the analysis is completed as directed in the method already described. The number of cc. of thiosulfate used multiplied by  $x$  (the value of 1 cc. of thiosulfate in terms of iodine) will give the excess of iodine present; and this subtracted from 50 will give the number of cc. of iodine consumed by the uric acid present. Dividing the mg. of uric acid by the cc. of iodine consumed will give the value of 1 cc. of the iodine solution in terms of uric acid.

3. *Calculation of an Unknown.*—Multiply the number of cc. of thiosulfate used by the value of  $x$  as determined in (1). This will give the excess of iodine present. Subtracting this value from 50 will give the number of cc. of iodine consumed. Multiplying the figure for the iodine consumed by the value of 1 cc. of iodine in terms of uric acid, found according to (2), will give the quantity of uric acid in mg. in 10 cc. of blood taken for analysis. If this figure be multiplied by 10, the uric acid content of 100 cc. of blood will thus be given.

#### SUMMARY.

1. An experimental study of a number of metallic salts as precipitants for uric acid in a solution alkaline with sodium carbonate was made. The results showed that nickel is the best of those tried.

2. A 0.0004 N iodine solution was found suitable for the estimation of small amounts of uric acid provided certain conditions are adhered to.

3. Based upon the above considerations, a new method has been developed for the determination of uric acid in blood, the chief features of which are: (a) The precipitation of the uric acid by means of nickel acetate in a solution alkaline with sodium carbonate. (b) The estimation of the uric acid in the precipitate by means of a dilute solution of iodine.

4. The method was applied with good results to aqueous solutions of uric acid as well as to blood serum to which known amounts of uric acid were added.

5. Low and inconsistent results were obtained when the method was applied to sheep's blood to which known amounts of uric acid were added. This was shown to be due to the inadequacy of the procedure generally employed for the coagulation and preliminary treatment of the blood. The colorimetric method when used in the analysis of samples of the same blood also gave low and inconsistent results for the same reason.

6. Comparison tests show that the volumetric method is fully as accurate as the colorimetric method, and possesses the advantage of requiring no special apparatus.

## THE NUTRITIVE VALUE OF THE BANANA.

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(Received for publication, August 20, 1918.)

In several of the tropical countries the banana, *Musa sapientum*, is a major foodstuff in the diet of the natives. Thus Adams<sup>1</sup> states, "that in the States of Parana and Santa Catarina, Brazil, the entire population subsists exclusively on bananas as a food, and coffee as a drink; and these sections are famous for the strength and endurance of their laboring classes." In the temperate zone, bananas are consumed only as a supplementary food.

Detailed studies of the nutritive value of the banana have not hitherto been reported. Prescott<sup>2</sup> in his recent article points out that the combination of bananas and milk, in proper proportion, would constitute a good diet for man. Loeb and Northrop<sup>3</sup> and Northrop<sup>4</sup> have shown that under aseptic conditions the fruit fly, *Drosophila ampelophila*, cannot be raised on bananas, but that the flies will grow on a mixture of yeast and bananas.

Myers and Rose<sup>5</sup> reported that the banana appears to be a particularly valuable food to employ in dietetic treatment in mild cases of nephritis with nitrogen retention.

The present work is a study of the nutritive value of bananas as determined by the maintenance and growth of albino rats when placed upon a diet of bananas, or bananas together with certain supplementary substances.

The general routine of the experiments was as follows: Young and vigorous albino rats, purchased from a reliable dealer, were

<sup>1</sup> Adams, F. U., *Conquest of the tropics*, 1914, 350.

<sup>2</sup> Prescott, S. C., *Scientific Monthly*, 1918, vi, 65.

<sup>3</sup> Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1916, xxvii, 309.

<sup>4</sup> Northrop, J. H., *J. Biol. Chem.*, 1917, xxx, 181.

<sup>5</sup> Myers, V. C., and Rose, A. R., *J. Am. Med. Assn.*, 1917, lxxviii, 1022.

used. The animals were kept in well ventilated round cages, about 10 inches in diameter, two or three animals being kept in each cage. The room in which the cages were kept was well lighted and a moderate temperature maintained. The animals were weighed each morning, after which fresh food was given. Distilled water was given *ad libitum*.

The various foods given were prepared as follows: The edible portion of well ripened, golden-yellow bananas was mashed alone, or with the supplementary substances indicated. The casein employed was prepared from commercial washed casein by boiling for 2 hours with 95 per cent alcohol; it was filtered and the casein washed with fresh alcohol, then allowed to dry in the air.

The water and the alcoholic extracts of carrots were prepared in the following manner. 700 gm. of fresh carrots, *Daucus carota*, were milled after removing as little as possible of the outer portion, and then treated with 1 liter of either distilled water or 95 per cent ethyl alcohol. They were allowed to stand for 48 hours in the water and for 5 days in the alcohol, with occasional shaking, at room temperature. Then the solution was filtered through a Buchner funnel, washed with fresh solvent, and concentrated *in vacuo* at low temperature, until the solution became syrupy. This syrupy liquid was kept in an ice box until used.

The salt mixture was prepared according to Osborne and Mendel.<sup>6</sup> Table butter was used as one of the sources of the fat-soluble accessory factor.<sup>7</sup> Brewers' yeast was filtered, pressed, dried in the air, and well ground.

It has been shown that an adequate diet must contain sufficient nitrogen, calories, and water, the salts, and yet unidentified substances: accessory factors,<sup>8</sup> oryzanin,<sup>9</sup> vitamine,<sup>10</sup> fat-soluble A and water-soluble B,<sup>7</sup> hormone,<sup>11</sup> or sitacoid.<sup>12</sup>

<sup>6</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 317, Salt Mixture IV.

<sup>7</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105.

<sup>8</sup> Hopkins, F. G., *Analyst*, 1906, xxxi, 391.

<sup>9</sup> Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89.

<sup>10</sup> Funk, C., *Ergebn. Physiol.*, 1912, xiii, 125.

<sup>11</sup> Mendel, L. B., in Lusk, G., *The elements of the science of nutrition*, 1917, Philadelphia, 378.

<sup>12</sup> Ramsden, W., *J. Soc. Chem. Ind.*, 1918, xxxvii, transaction 53.

In the first experiment the rats were fed upon bananas exclusively. Ration 1: Bananas, 100 per cent. The results obtained from this experiment are given in Chart 1, from which it is clearly seen that the rats failed to grow on the banana ration. Some animals maintained their weight and lived for about 80 days, while others died in a short time. The rats lose from 5 to 10 gm. on the 1st day that the change is made from an adequate diet, bread and carrots, to the all banana diet. Following the 1st day's drop in weight and until death, the body weight

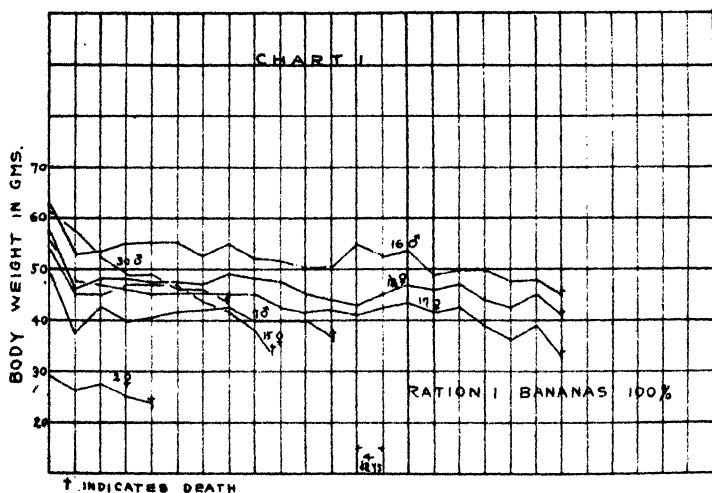


CHART 1.

of the animals remained almost unchanged. The animals appeared to be in poor condition; some developed an eye infection, and some rats (Nos. 16, 17, and 18) developed scurvy in about 30 days. The lack of growth and final death of all the animals fed exclusively upon bananas shows that this fruit is deficient in one or more necessary food factor.

A comparison of the chemical composition of the banana with a few other common foodstuffs (Tables I and II) is of interest in connection with this investigation, and may serve to throw some light upon the nature of the deficiency in the banana.

Wheat bread soaked in whole milk or supplemented with fresh vegetables, or occasionally with small amounts of meat, is recognized as an adequate diet for the albino rat. Bread constitutes the principal part of such a diet. A glance at these analyses will make it plain that bananas contain only one-half as much fat and carbohydrate, and about one-seventh as much protein as the bread, while the ash content is nearly the same in both cases. Aside from these chemical differences, the digestibility and the adaptability of the bananas might be considered.

TABLE I

Food.*	Water.	Ash.	Protein (N × 6.25)	Fat.	Carbo- hydrate.	Fuel value, per pound.
	per cent	per cent	per cent	per cent	per cent	calories
Bananas (edible).....	75.3	0.8	1.3	0.6	22.0	447
Potatoes (white).....	78.3	1.0	2.2	0.1	18.4	378
Carrots (fresh edible)....	88.2	1.0	1.1	0.4	9.3	204
Oatmeal.....	7.3	1.9	16.1	7.2	67.5	1,811
Bread (white).....	35.3	1.1	9.2	1.3	53.1	1,199

\* Sherman, H. C., Chemistry of food and nutrition, New York, 1918.

TABLE II.

*Ash Constituents of Foods in Gm. per 100 Calories of Edible Food Material.\**

Food.	CaO	MgO	K <sub>2</sub> O	Na <sub>2</sub> O	P <sub>2</sub> O <sub>5</sub>	Cl	S	Fe
Bananas.....	0.01	0.04	0.50	0.02	0.055	0.20	0.013	0.0006
Bread.....	0.011	0.011	0.04		0.075		0.05	0.0003
Carrots.....	0.168	0.074	0.765	0.28	0.22	0.078	0.048	0.0016

\* Sherman, H. C., Chemistry of food and nutrition, New York, 1918.

Starch in the banana is largely converted into sugar as the fruit ripens. It is easily digested when ripe, but it would seem possible that the physical condition of the starch and sugar in the banana may not be suited to the digestive tract of the rat.

The second experiments were carried out by supplementing some of the probable missing substances in the banana.

Charts 2 and 3 show the results obtained from adding small amounts of yeast preparation or butter fat, which contain two different kinds of food hormones.

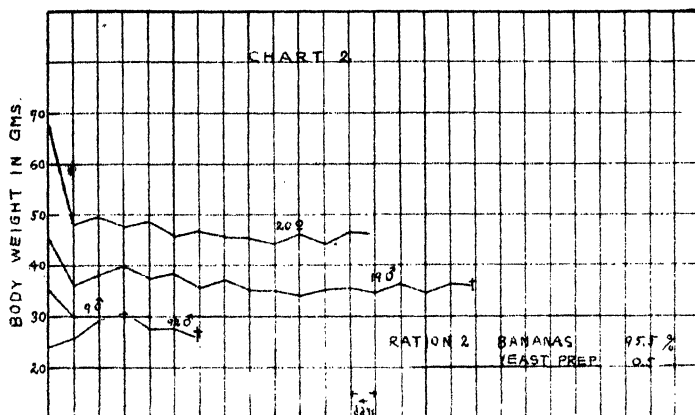


CHART 2.

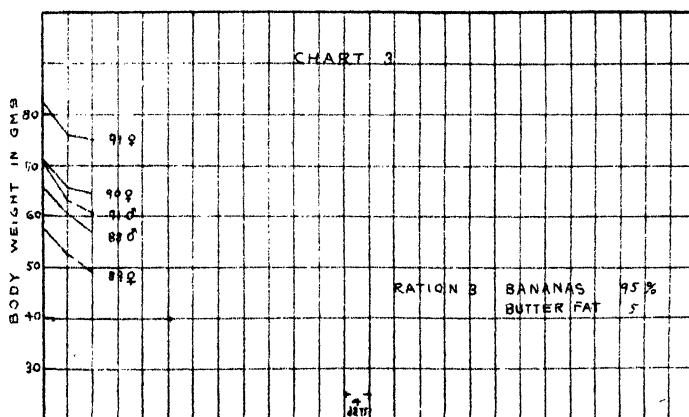


CHART 3.



*Ration 2.*

	<i>per cent</i>
Bananas.....	95.5
Yeast preparation.....	0.5

*Ration 3.*

Bananas.....	95
Butter fat.....	5

One of the absolutely necessary elements to maintain the normal condition of the tissues is the presence of certain salts. Chart 4 indicates the results obtained by adding a complete salt mixture to the banana ration. Chart 5 shows the results obtained by adding milled fresh carrots.<sup>13</sup>

*Ration 4.*

	<i>per cent</i>
Bananas.....	96.3
Salt mixture.....	3.7

*Ration 5.*

	<i>per cent</i>
Bananas.....	50
Carrots.....	50

From the preceding experiments we reach the following conclusions. Neither the yeast preparation, which contains water-soluble B, nor the butter fat, which contains fat-soluble A, show any complementary action for the banana. The addition of the salt mixture to the banana diet resulted in no growth of the animals. The addition of carrots likewise produced no growth.

Thus far our experiments show that the banana is not an adequate diet for rats. Therefore, the next problem is to find whether the failure of growth in the animals was due to a deficiency in protein or to this deficiency and some other in addition. The following rations were employed:

<sup>13</sup> Tables I and II show that the composition of bananas and carrots is almost similar, except for carbohydrates, yet young rats live on a banana diet relatively much longer than on a carrot diet. It is believed that the difference in the nutrition of young animals when fed upon these closely allied forms of food is due to a difference in the degree of digestibility.

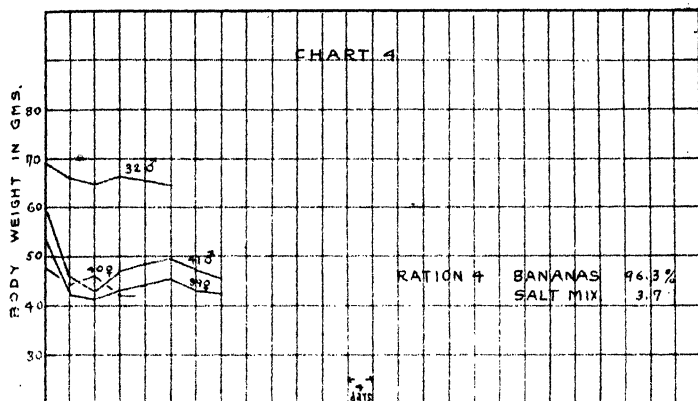


CHART 4.

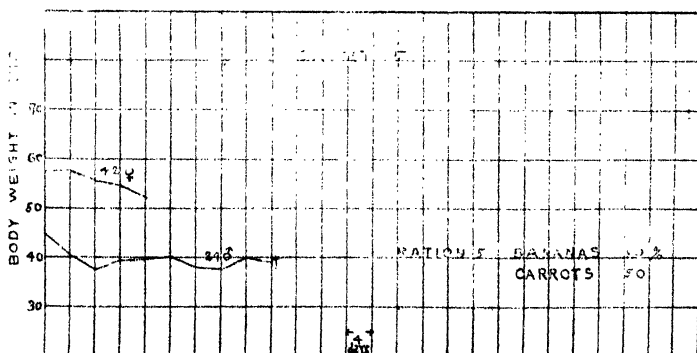


CHART 5.

*Ration 6.*

	<i>per cent</i>
Bananas.....	90.8
Yeast preparation.....	0.5
Butter fat.....	5.0
Salt mixture.....	3.7

*Ration 7.*

Bananas.....	90.8
Extract of carrots.....	0.5
Butter fat.....	5.0
Salt mixture.....	3.7

*Ration 8.*

Bananas.....	84
Casein.....	16

*Ration 9.*

Bananas.....	79
Casein.....	16
Butter fat.....	5

*Ration 10.*

Bananas.....	75.3
Casein.....	16.0
Butter fat.....	5.0
Salt mixture.....	3.7

*Ration 11.*

Bananas.....	75.3
Purified beef.....	16.0
Butter fat.....	5.0
Salt mixture.....	3.7

*Ration 12.*

Bananas.....	59.0
Purified beef.....	16.0
Butter fat.....	5.0
Salt mixture.....	3.7
Carrots.....	16.3

*Ration 13.*

Bananas.....	59.0
Casein.....	16.0
Butter fat.....	5.0
Salt mixture.....	3.7
Carrots.....	16.3

*Ration 14.*

	<i>per cent</i>
Bananas.....	74.8
Casein.....	16.0
Butter fat.....	5.0
Salt mixture.....	3.7
Water extract of carrots.....	0.5

*Ration 15.*

Bananas.....	74.8
Casein.....	16.0
Butter fat.....	5.0
Salt mixture.....	3.7
Alcoholic extract of carrots.....	0.5

*Ration 16.*

Bananas.....	83.5
Casein.....	16.0
Water extract of carrots.....	0.5

*Ration 17.*

Bananas.....	83.5
Casein.....	16.0
Alcoholic extract of carrots.....	0.5

*Ration 18.*

Bananas.....	83.5
Casein.....	16.0
*Extract of carrots.....	0.5

\* For explanation, see p. 182.

*Ration 19.*

Bananas.....	74.8
Casein.....	16.0
Butter fat.....	5.0
Salt mixture.....	3.7
Yeast preparation.....	0.5

*Ration 20.*

Bananas.....	79.8
Casein.....	16.0
Salt mixture.....	3.7
Yeast preparation.....	0.5

*Ration 21.*

Bananas.....	83.5
Casein.....	16.0
Yeast preparation.....	0.5

## DISCUSSION.

In the first part of our experiments we have shown that bananas alone, or bananas supplemented with either the yeast preparation, butter fat, salt mixture, or carrots did not produce growth of young rats.

Then young rats were fed with rations made up of bananas, yeast preparation, butter fat, and salt mixture or extract of carrots (both water and alcoholic), in the place of the yeast preparation. These rations are Nos. 6 and 7, and some of the results are shown in Chart 6. The animals did not thrive on this diet.

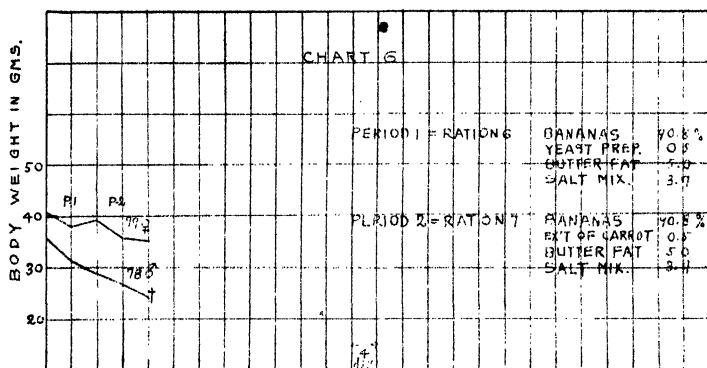


CHART 6.

The addition of 16 per cent of purified casein to the banana diet, in some cases, had a marked influence upon the growth of the rats, while in others there was about one-third of normal growth, and still others did not grow at all (Chart 7, Ration 8).

If washed casein is boiled with 95 per cent ethyl alcohol for 2 hours, cooled, filtered, and washed well with fresh alcohol, it is said that the purified product is free from food hormones. Absolute ethyl alcohol does not remove the food hormones from dried foodstuffs, which contained food accessory substances. Osborne and Wakeman<sup>14</sup> have isolated a new kind of protein from

<sup>14</sup> Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xx xiii, 7, 243.

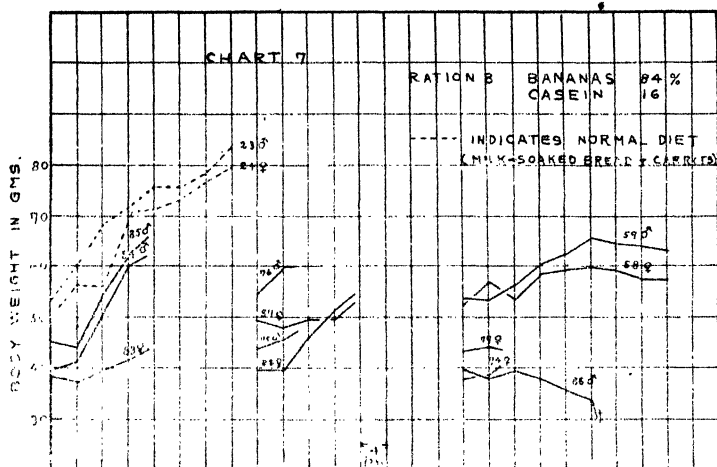


CHART 7.

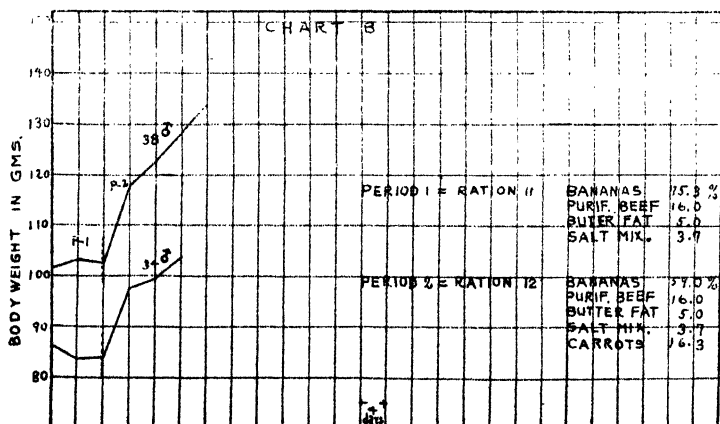


CHART 8.

the alcoholic washings of casein. Whether the presence of this new protein in the casein has any connection with the growth-promoting factors or not we cannot state definitely at present.

Substituting an equal quantity of purified beef<sup>15</sup> for purified casein shows that this diet does not cause the growth of young rats. However, upon adding a definite quantity of carrots, Ration 12, to the same diet we obtained normal growth (Chart 8).

Instead of whole fresh carrots we added a small quantity of water and alcoholic extracts of fresh carrots to the diets, either made up of bananas, casein, butter fat, and salt mixture or bananas and casein (Rations 14 to 17). Since both water and alcoholic extracts of carrots possessed an equal amount of growth-promoting substances, we designated them as extract of carrots for the sake of simplicity (Ration 18). Both water and alcoholic extracts of carrots possess growth-promoting substances, as shown in Charts 9 to 12, and 15 and 16.

Our experiments have shown (Charts 7, 9, 10, 11, 12, 15, and 16) that the banana contains enough fat, carbohydrate, and inorganic salts, but is deficient in protein, and in at least one food hormone (water-soluble B).

A second aqueous or alcoholic extract of carrots, which yielded about 12 per cent of the first product, also contained food hormones. Aluminium hydroxide and colloidal iron removed the food hormones from both the aqueous and alcoholic extracts of carrots.

A detailed account of the preparation, isolation, and analyses of the water and alcoholic extract of carrots will be given in a later paper.

Chart 9 shows the action of animal growth in the presence and absence of the growth-promoting substances in different rations given in the text. This set is an example of many similar experiments carried out with different rations.

<sup>15</sup> Fresh beef was ground finely in a meat chopper and about 250 gm. were treated with 400 cc. of water and boiled for 10 minutes. The meat then was filtered through muslin while hot, washed with hot water, and squeezed. This treatment was repeated. Then the meat was treated with 300 cc. of water, placed in a large collodion bag, and after adding a little toluene it was dialyzed for 2 days in cold running water. At the end of the period the mixture was filtered through muslin and the above treatment repeated. Finally the purified beef was dried in a vacuum desiccator over sulfuric acid.

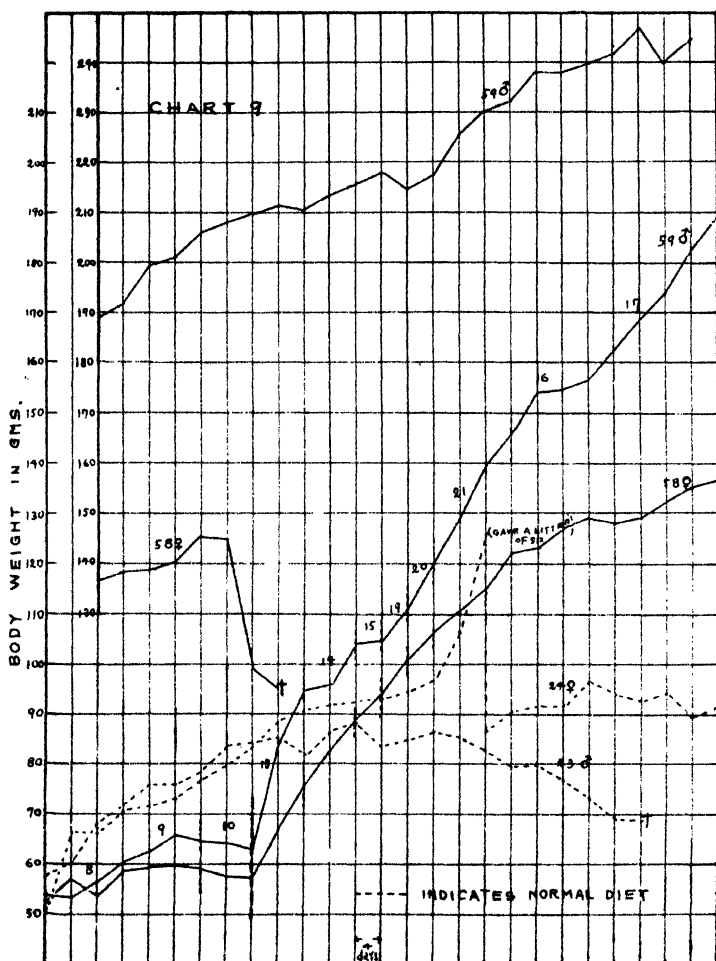
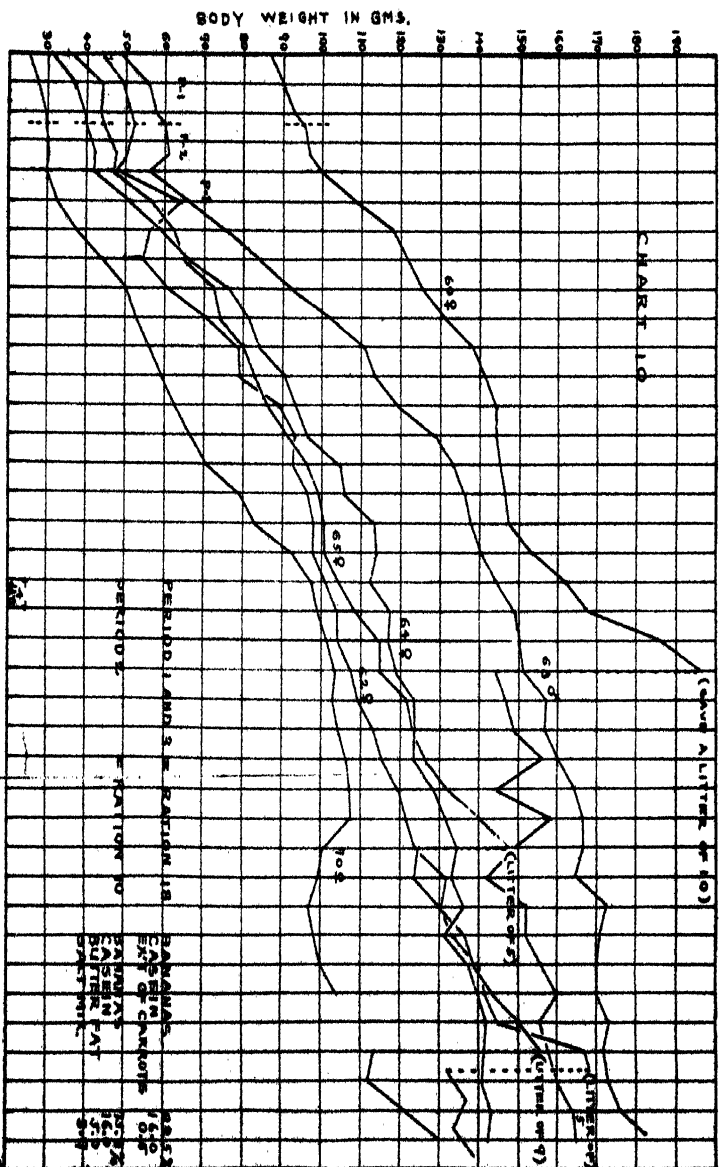


CHART 9. The figures represent the number of the ration. Curves to the left of the heavy vertical line indicate the growth of rats fed upon the diets free from the growth-promoting substances, while the curves to the right of this line indicate growth of rats fed upon the diets which contained the growth-promoting substances. The broken lines represent the growth of animals fed upon a diet of bread, soaked in whole milk, and carrots.

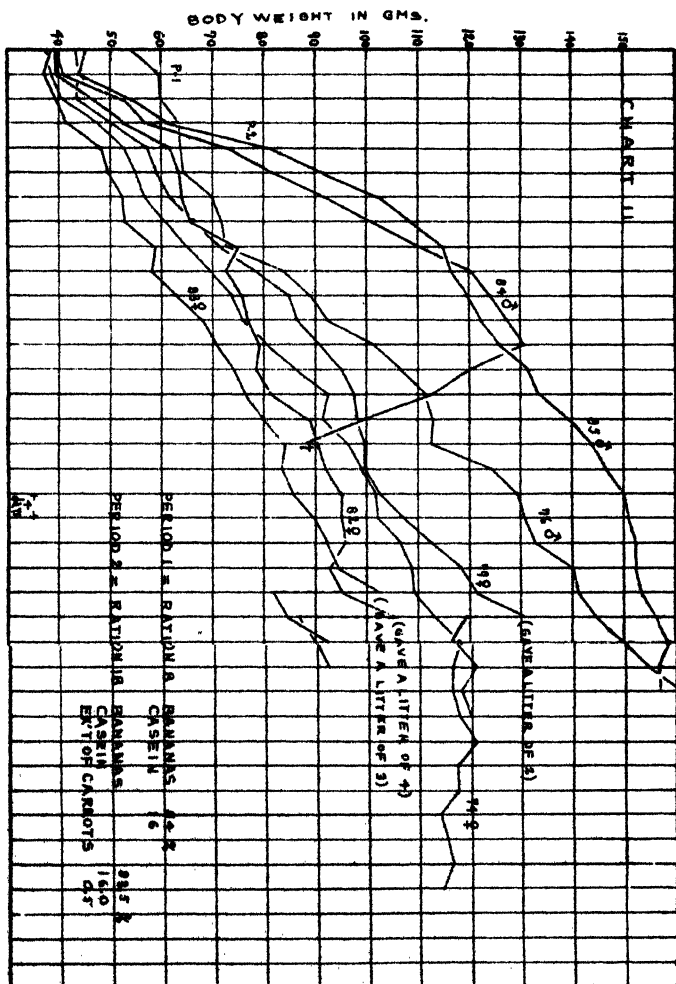




### CHART 10.







### CHART 11.



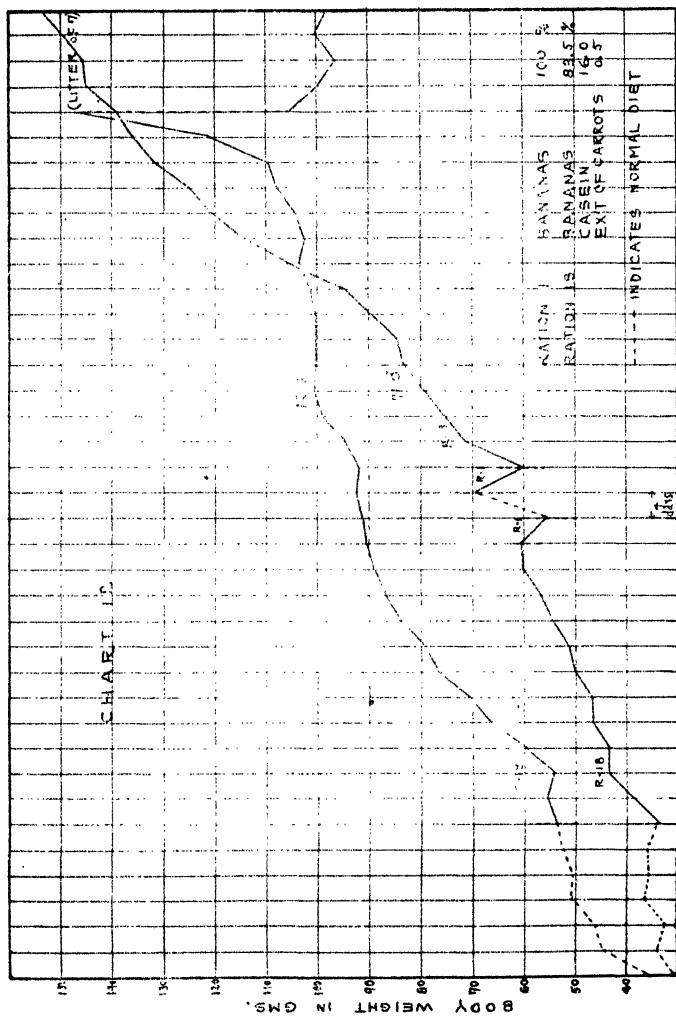


CHART 12.

## Nutritive Value of Banana

Young rats were removed from their mothers at the age of 25 days, or before, and allowed to live independently on Ration 18. Charts 13, 14, 15, and 16 show that most of them did not main-

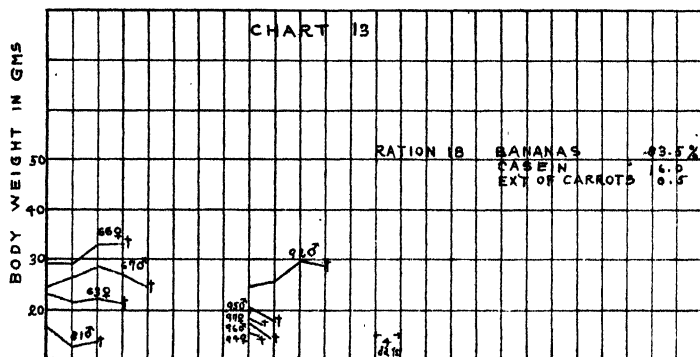


CHART 13.

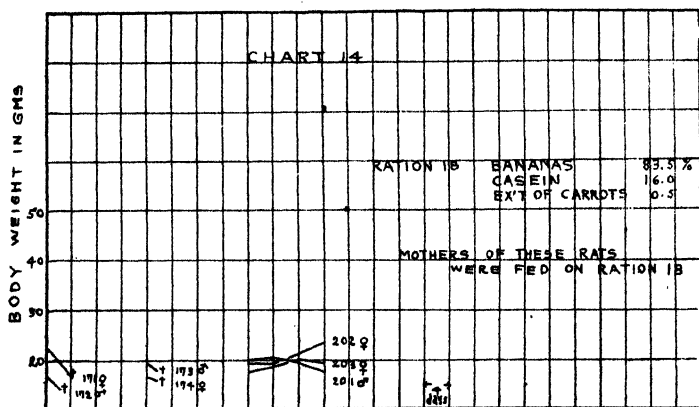


CHART 14.

tain growth and life, while a few animals grew more rapidly than normally.

Further experiments, continued for longer periods of time, have clearly shown that the banana plus purified casein and carrot

extract (or yeast preparation) constitutes a complete diet for the growth, maintenance, and reproduction of the albino rat.

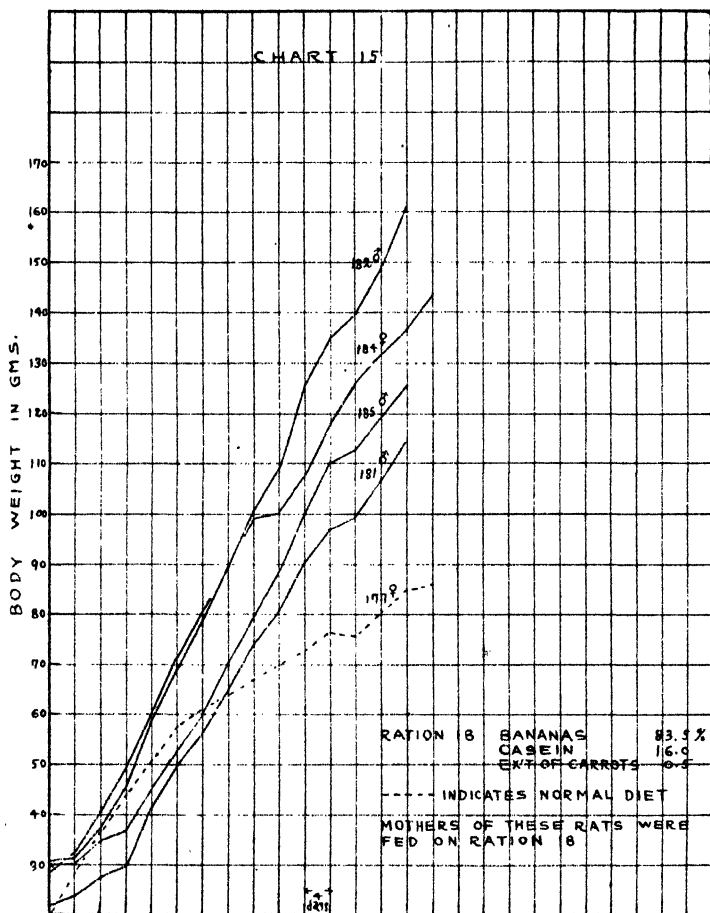


CHART 15.

Numerous litters of from 2 to 10 rats have been born of parents fed solely upon Ration 18 for months. The puberty and gestation period of the females fed on Ration 18, and the aver-



age individual birth weight and the sex ratio of the litters born of parents fed solely upon Ration 18 for months corresponds almost in every respect to rats fed on normal bread diet, as reported by Donaldson.<sup>16</sup> During the period of lactation, it appears, however, that it is impossible to provide proper nutrient for the new-born, when the mother is limited solely to the above mentioned ration. The addition of milk to the diet of the mother from the time of birth of the young until they are weaned is absolutely imperative, if the young are to survive.<sup>17</sup> Repeated

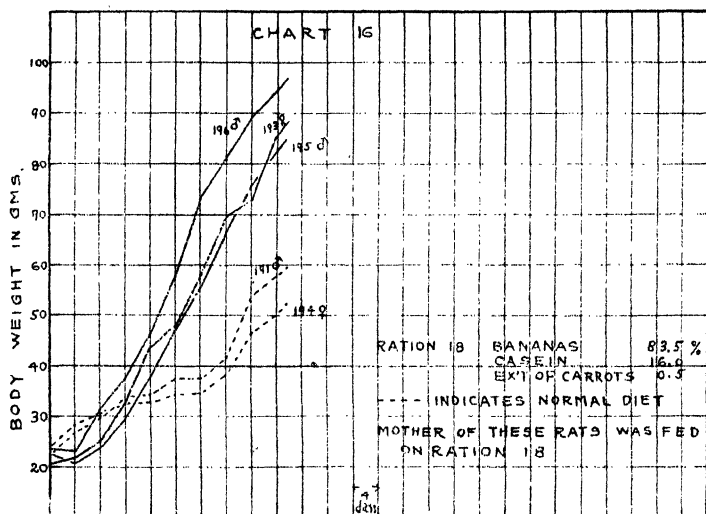


CHART 16.

trials have rendered this conclusion unquestionable. Yet as soon as the young are given food other than the mother's milk, limiting both mother and young to the banana-casein-carrot extract ration, a rapid and very satisfactory growth on the part of the young follows. With the exception of the break in the

<sup>16</sup> Donaldson, H. H., *The Rat*, Memoirs of The Wistar Institute of Anatomy and Biology, No. 6, Philadelphia, 1915, 21, 24, 27.

<sup>17</sup> Further investigation on this particular point will be given in a later paper.

mother's diet during the period of lactation, rats of the second generation have been brought to maturity solely upon Ration 18. The young rats of the second generation grow more rapidly on the limited ration than do those of the first. This is possibly due to the fact that care in the laboratory has produced stronger animals than those secured from the dealer. Certainly these animals have grown very rapidly upon Ration 18 to complete maturity (see Charts 15 and 16), and their general condition has been most excellent.

#### CONCLUSIONS.

1. The banana is deficient in (a) protein and (b) the water-soluble accessory as a foodstuff for the growth or maintenance of albino rats.

2. A diet of bananas, purified casein, and yeast or carrot-extract is sufficient for the perfect growth and reproduction of the albino rat. Such a diet is not, however, adequate for the production of proper milk by the mother. This statement holds true whether the litter of young is large or small in number, showing that the failure in milk is qualitative in nature, and not simply a deficiency in the quantity of milk product. It is interesting to observe that a diet can be adequate for the production of young and for growth after the eyes of the young open, but inadequate for the proper production of milk.

3. Purified casein supplements the banana other than by the simple addition of protein. The casein in the ration cannot be satisfactorily replaced by washed and dialyzed beef.



# A PRELIMINARY REPORT ON THE PREPARATION OF ANTIPOLYNEURITIC SUBSTANCES FROM CARROTS AND YEAST.

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the Waldo Story Research Fund, Harriman Research Laboratory,  
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(Received for publication, August 20, 1918.)

Natural curative substances for polyneuritis induced in pigeons and fowls by a diet of polished rice have been prepared from rice polishings,<sup>1-15</sup> autolyzed yeast,<sup>4, 8, 15-18</sup> animal tissues,<sup>11, 19</sup> cab-

<sup>1</sup> Eijkman, C., *Virchows Arch. path. Anat.*, 1897, cxlviii, 523; cxlix, 187.

<sup>2</sup> Fraser, H., and Stanton, A. T., *Philippine J. Sc., B.*, 1910, v, 55.

<sup>3</sup> Chamberlain, W. P., and Vedder, E. B., *Philippine J. Sc., B*, 1911, vi, 251.

<sup>4</sup> Chamberlain, W. P., and Vedder, E. B., *Philippine J. Sc., B*, 1911, vi, 395.

<sup>5</sup> Funk, C., *J. Physiol.*, 1911-12, xliii, 395.

<sup>6</sup> Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89.

<sup>7</sup> Chamberlain, W. P., Vedder, E. B., and Williams, R. R., *Philippine J. Sc., B*, 1912, vii, 39.

<sup>8</sup> Edie, E. S., Evans, W. H., Moore, B., Simpson, G. C. E., and Webster, A., *Biochem. J.*, 1912, vi, 234.

<sup>9</sup> Fraser, H., and Stanton, A. T., *Tr. 15th Internat. Cong. Hyg. and Demog.*, 1913, v, 637.

<sup>10</sup> Strong, R. P., and Crowell, B. C., *Tr. 15th Internat. Cong. Hyg. and Demog.*, 1913, v, 679.

<sup>11</sup> Cooper, E. A., *Biochem. J.*, 1912-13, vii, 268.

<sup>12</sup> Vedder, E. B., *Tr. 15th Internat. Cong. Hyg. and Demog.*, 1913, v, 671.

<sup>13</sup> Drummond, J. C., and Funk, C., *Biochem. J.*, 1914, viii, 598.

<sup>14</sup> Funk, C., *Biochem. Bull.*, 1915, iv, 304.

<sup>15</sup> Funk, C., *Biochem. Bull.*, 1916, v, 1.

<sup>16</sup> Cooper, E. A., and Funk, C., *Lancet*, 1911, ii, 1266.

<sup>17</sup> Funk, C., *J. Physiol.*, 1912-13, xlv, 75.

<sup>18</sup> Cooper, *Biochem. J.*, 1914, viii, 250.

<sup>19</sup> Cooper, *J. Hyg.*, 1914, xiv, 12.

bage and potato juices,<sup>20</sup> and egg yolk.<sup>19, 21</sup> Painsstaking efforts have been made during the last 10 years by many scientists to isolate the substances, in order to establish the chemical nature of these curative materials. During the last 2 or 3 years artificial antipolyneuritic substances have been prepared and discussed by Funk,<sup>22</sup> Williams,<sup>23</sup> Williams and Seidell,<sup>24</sup> and Williams.<sup>25</sup> However, Voegtlin and White,<sup>21</sup> Steenbock,<sup>21</sup> and Harden and Zilva<sup>27</sup> do not confirm their results.

In a preceding paper,<sup>28</sup> we have shown that both water and alcoholic extracts of fresh carrots<sup>29</sup> contain growth-promoting substances. It was further found that our yeast preparations showed the same growth-promoting power exhibited by the carrot extracts.

#### EXPERIMENTAL.

The water and the alcoholic extracts of carrots were prepared in the following manner. Fresh carrots were finely ground in a meat chopper after removing as little as possible of the outer coating, since nutrient materials are stored in the thin-walled parenchymatous bast. 700 gm. were treated with 1 liter of distilled water and allowed to stand for 2 days at room temperature with occasional shaking, or were treated with 1 liter of 95 per cent ethyl alcohol for 5 days. In either case the mixture was filtered through a hardened paper in Buchner funnel, washed with fresh solvent, and the clear golden-yellow filtrate concentrated *in vacuo* at low temperature, until it became a syrupy mass. This was kept in a refrigerator for future use.

<sup>20</sup> McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

<sup>21</sup> Steenbock, H., *J. Biol. Chem.*, 1917, xxix, p. xxvii.

<sup>22</sup> Funk, C., *J. Physiol.*, 1912-13, xlv, 489.

<sup>23</sup> Williams, R. R., *J. Biol. Chem.*, 1916, xxv, 437.

<sup>24</sup> Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

<sup>25</sup> Williams, *J. Biol. Chem.*, 1917, xxix, 495.

<sup>26</sup> Voegtlin, C., and White, G. F., *J. Pharm. and Exp. Therap.*, 1917, ix, 155.

<sup>27</sup> Harden, A., and Zilva, S. S., *Biochem. J.*, 1917, xi, 172.

<sup>28</sup> Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 171.

<sup>29</sup> It may be of interest to note that it has been a custom among inhabitants in the Province of Owari, Japan, to feed a racing horse with a couple of fresh carrots just prior to the start of a race. Natives say that fresh carrots make the animal more lively.

The chemical nature and yield of the substances varies somewhat with the time and the temperature at which extractions are made, and with the concentration and the variety of carrots. A chemical analysis of both extracts, using the above technique and concentration, may be of interest in connection with this investigation. The total nitrogen in the moist preparations was estimated by the Kjeldahl method. The total phosphorus was precipitated as ammonium phosphomolybdate in presence of sulfuric acid.<sup>30</sup> The micro-apparatus of Van Slyke<sup>31</sup> was used for the determination of amino nitrogen without hydrolysis with acid. Preparations were tested with Folin's uric acid<sup>32</sup> and phenol<sup>33</sup> reagents.

Both preparations had a sweet taste. Their yields were about 7 per cent of original fresh carrots.

Table I shows the analysis of preparations. The results are expressed in percentage of substances calculated on a moisture-free basis.

TABLE I.

Substance.	Total nitrogen	Total ash.	P <sub>2</sub> O <sub>5</sub>	Amino nitrogen.	Uric acid.	Phenol.
	per cent	per cent	per cent	per cent	per cent	per cent
Water extract of carrots.....	1.51	7.10	0.91	1.09	0.17	0.55
Alcoholic extract of carrots.....	0.44	3.17	0.21	0.31	0.02	0.56

In connection with this analysis it is of interest to note that both water and alcoholic extracts have about the same amount of phenol-reacting substance, and that both extracts are equally efficient in their growth-promoting power.

A colorless crystalline substance from yeast was prepared in the following manner. Brewers' yeast was filtered, pressed, dried openly in the air, and well ground. 10 gm. of powdered yeast preparation, the nitrogen content of which was 9.59 per cent, were treated with 100 cc. of distilled water, shaken well, and placed in a 300 cc. collodion bag, which was suspended in air for

<sup>30</sup> Falk, K. G., and Sugiura, K., *J. Am. Chem. Soc.*, 1915, xxxvii, 1507.

<sup>31</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 121.

<sup>32</sup> Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912-13, xiii, 363.

<sup>33</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.

air dialysis<sup>34</sup> at room temperature, until the contents of the bag became perfectly dry. The time required for this process was about 10 days. The colorless crystalline substance on the outer wall of the bag was carefully removed by means of a camel's hair brush. By this method about 5 mg. of crystalline substance were obtained from 10 gm. of yeast preparation. Occasionally no crystals separated on the outer wall of the bag.

A quantitative chemical study of this substance was not made because we have not yet obtained sufficient material. Some of the qualitative tests upon the crystals might be given here. The crystals were in plate form, and were not easily soluble in water. When 5 mg. of substance in water were treated with phosphomolybdic-phosphotungstic reagent, a deep blue color was produced; but no color reaction was given with the phosphotungstic acid reagent.

A colorless crystalline substance was also prepared in the following manner. 10 gm. of powdered yeast preparation were treated with 100 cc. of 5 per cent sodium chloride solution, and after being placed in a collodion bag, were subjected to air dialysis, as before. By this method we obtained 22 mg. of colorless crystalline substance, which was almost free from sodium chloride.

Polyneuritis in pigeons was induced by feeding them *ad libitum* on white rice and water. The pigeons were kept upon the same diet after treatment, unless otherwise mentioned. All the injections were given subcutaneously into the breast.

*Experiment 1.*—On the 18th day, Pigeon 5 showed symptoms of polyneuritis. The bird rolled about in the cage with paralysis of the legs and retracted neck. 2.4 mg. of crystals from the yeast preparation in 1.5 cc. of water were administered. Within 1 hour the pigeon was able to stand upright and the neck was no longer retracted. On the 2nd day after injection, the condition of the pigeon was almost normal, and the diet was changed to normal and kept so for 11 days. Her condition was good. Then normal diet was changed to white rice again. On the 23rd day, the bird showed marked symptoms of polyneuritis. 37.5 mg. of alcoholic extract of carrots in 1 cc. of water were administered. Within 1 hour the bird regained its strength, but the disease reappeared the next day and it died the following night.

*Experiment 2.*—On the 20th day, Pigeon 6 showed the characteristic symptoms of polyneuritis and was given 5 mg. of crystals from the yeast

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<sup>34</sup> Kober, P. A., *J. Am. Chem. Soc.*, 1917, xxxix, 944.

preparation in 1.5 cc. of water. Within 1 hour it regained strength, and on the following day appeared almost in normal condition. Then the bird was fed with normal diet for 10 days, after which a rice diet was given. On the 34th day, symptoms returned, and 52.7 mg. of water extract of carrots in 1.5 cc. of water were administered. On the 2nd day, the condition of the pigeon was a little better, but it died on the 4th day without marked improvement in the meantime.

*Experiment 3.*—On the 20th day, Pigeon 4 showed symptoms of polyneuritis and 69 mg. of water extract of carrots in 1.5 cc. of water were administered. Within 30 minutes the bird was practically able to stand upright and walk, and its neck was no longer retracted. For 10 days from the following day normal diet was fed; then the diet was changed again to white rice and water. On the 22nd day, paralysis again appeared, and 34.5 mg. of water extract of carrots in 1 cc. of water were injected. On the following day the condition was improved, but the neck remained retracted. On the 2nd day, the bird appeared in fine condition; the neck was no longer retracted. On the 3rd day, it was in almost a normal condition. On the 11th day the disease reappeared, and 69 mg. of water extract of carrots in 1 cc. of water were administered. During the next 2 days the pigeon improved and was able to walk upright, but died on the 3rd day.

*Experiment 4.*—On the 42nd day, Pigeon 1 showed the recognized symptoms, and 52.7 mg. of water extract of carrots in 1.5 cc. of water were injected. The bird died within 10 minutes, without any sign of improvement.

*Experiment 5.*—On the 36th day, Pigeon 2 showed the symptoms of polyneuritis. 2.4 mg. of crystals from yeast preparation in 1.5 cc. of water were administered. The bird died 2 days later without showing much improvement.

*Experiment 6.*—On the 35th day, Pigeon 3 showed symptoms of polyneuritis. 124 mg. of alcoholic extract of carrots in 1 cc. of water were injected, but the bird died during the night.

*Experiment 7.*—On the 19th day, Pigeon 7 showed symptoms of polyneuritis, with partial paralysis of the legs and retraction of neck, and 52.7 mg. of water extract of carrots in 1.5 cc. of water were administered. On the following day, the bird appeared to be in better condition. On the second day, it was almost normal, and the neck was no longer retracted. The disease reappeared on the 21st day after the first injection. At 1 p.m., 52.7 mg. of water extract of carrots in 1.5 cc. of water were administered. The bird died during the night without having showed any signs of improvement.

*Experiment 8.*—On the 43rd day, Pigeon 8 showed the characteristic symptoms of polyneuritis, and 75 mg. of alcoholic extract of carrots in 1 cc. of water were administered. On the same day the condition was much better, but the bird died on the 2nd day without further improvement.

*Experiment 9.*—On the 32nd day, Pigeon 14 rolled about in the cage with marked paralysis of legs. 87 mg. of alcoholic extract of carrots in 1.5 cc. of water were injected. On the following day, the condition re-



mained the same. On the 3rd day, 3.5 mg. of crystals from the yeast preparation in 1.5 cc. of water were administered, but the bird died during the night.

#### DISCUSSION AND CONCLUSIONS.

Our experimental results are not extensive, but they show clearly that the extracts of fresh carrots and crystalline substances from yeast preparation may cure polyneuritis in pigeons in those cases where the disease has developed quickly; namely, in about 20 days. However, these same substances did not prevent the polyneuritic pigeons from death, if the symptoms appeared more slowly.

In this connection we quote a statement of Williams,<sup>23</sup> since our observations are quite similar to his:

Since forced feeding was not resorted to on account of the time necessary to care for a large number of birds in this way, it was occasionally the case that pigeons became weak and emaciated before the characteristic symptoms developed to the desired point. Such cases are not of course strictly comparable with those of sudden development in which the animals retain a great deal of their original strength and vigor, though losing muscular control, and a measure of uncertainty is thereby introduced in occasional cases.

The writer wishes to express his great appreciation to Dr. Stanley R. Benedict of the Cornell University Medical College and Miss Ruth C. Theis of Memorial Hospital, New York, for their helpful advice and suggestions in carrying out these experiments.

## THE DIETARY PROPERTIES OF THE POTATO.

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(Received for publication, August 22, 1918.)

### *Dietary Properties of Potato.*

The potato yields considerably less per acre than do most of the common root crops. Carrots and beets usually produce about twice as much dry matter, and turnips and parsnips at least a third more. The potato, which is morphologically an underground stem, has nevertheless found much greater favor as a human food than any of the root crops mentioned. This has been attributed, and probably correctly, to the lack of flavor in the potato, which makes it possible to confer palatability upon it by the addition of milk, butter, and cream, salt and pepper, or by frying in fats. All the edible roots commonly used as human foods in America possess pronounced flavors, and these do not seem to appeal to the appetite when regularly used in the diet. The sweet potato, which has little flavor other than sweetness, is the nearest competitor of the white potato. On teleological grounds it might be assumed that the special favor in which this tuber is held, is the result of some special value which it possesses from a nutritive standpoint, which has been unconsciously recognized by man in the course of his long familiarity with the two types of potatoes.

There are indeed some experimental data which seem to indicate that the nitrogen-containing compounds of the potato possess extraordinary value as a source of the cleavage products of protein. Hindhede (1) reports having maintained nitrogen equilibrium in men during periods as long as 27 to 97 days with a diet consisting solely of potatoes and margarine (reported to be practically nitrogen-free). There were very small additions of other

articles to the diet but they were so small and irregular that they were left out of consideration by the author.

Thomas (2) attempted, by experiments on an adult, to determine the relative biological values of the proteins of a number of the more important human foods. A critical examination of his protocols fully justifies the conclusion of Rose and Cooper (3) that the evidence does not warrant the acceptance of his values as an actual quantitative comparison of the foods he employed. Thomas asserts that in man the proteins of milk and of meat can replace the tissue protein lost through metabolic processes, to the extent of about 100 per cent. Maize and wheat proteins in the mixtures in which they occur in the kernels, were found by Thomas to be capable of replacing respectively 29.5 and 39.5 per cent of their weight of tissue proteins. On the same scale he assigned to the nitrogen of the potato a value of 83, indicating the belief that potato nitrogen has about twice the value of the nitrogen of wheat.

Rose and Cooper (3) have amply confirmed these results with respect to the potato, in a well controlled experiment in which a young woman remained in nitrogen equilibrium during a 10 day period on a diet consisting solely of potato, butter fat, and sugar. The subject weighed 50 kilos, and took during the first 5 days 4.52 gm. and during the last 5 days 4.97 gm. of nitrogen daily, this being derived entirely from potato. The results of the investigators mentioned are all in harmony in ascribing to the nitrogen of the potato a biological value considerably greater than that found by McCollum and his coworkers for any of the protein mixtures contained in the more important seeds employed as human foodstuffs.

We have made a study of the dietary properties of the white (Irish) potato by the method which we have employed during the last 3 years for the biological analysis of the foodstuffs. This consists of feeding the single natural food alone, and with single and multiple additions of purified food substances; *viz.*, inorganic salts, protein, and fat-soluble A. Our experiments relate to the properties of the potato as a food for the young rat during the growing period, as contrasted with those of the other investigations referred to, in all of which the problem involved the maintenance of nitrogen equilibrium in the adult.

The results of our studies are described in Charts 1 to 7. They show that the dietary properties of the potato closely resemble those of the cereal grains. In this tuber the first limiting factor for growth is the relative shortage of the elements, calcium, sodium, and chlorine, as has been found to be the case with the seeds thus far examined. The content of fat-soluble A is too low for the promotion of nutrition at the optimum, and the biological value of the nitrogen which it yields seems to be of the same order as that of the cereal grains. Our results on the value of the potato nitrogen *for growth* place it in a very different light from those reported by other investigators who have observed its value for the maintenance of nitrogen equilibrium in the adult.

In those experiments in which the diets supplied protein ( $N \times 6.25$ ) equal to 7 to 8 per cent of the food mixture, and this all derived from the potato, and the other dietary factors were approximately satisfactorily adjusted, growth took place very slowly or not at all, individual animals showing somewhat different capacity to make use of the diet (Chart 3, Lot 1283). We have elsewhere described about the same rate of growth in young rats, fed a diet which was fairly satisfactorily constituted in every respect, except in the protein factor, the content of the latter being but 7 per cent of the food mixture and derived solely from the maize kernel (4) and from the wheat kernel (5) respectively. That the quality of the protein in all these diets was the limiting factor was demonstrated by other experiments in which the diets were similar in every respect except that a portion of the carbohydrate was replaced by the purified protein casein. This modification of the diets led to rapid growth, and the appearance of every indication of good nutrition in the animals. It seems necessary to conclude from these results that *for growth* the nitrogen of the potato is of no greater value than an equivalent amount of protein from one of these cereal grains. This conclusion necessitates the assumption that there is nothing in the potato which exerts an appreciable depressing effect on the animals, a possibility which we have not entirely eliminated by our experimental procedure. There is, however, good reason to believe that a high consumption of potato does not cause marked injury to growing animals when fed continuously over a long period.

The inorganic content of the potato differs from that of the cereal grains in marked degree only in respect to potassium. The analyses of Forbes (6) show 1.89 per cent of K in dry potato, as against 0.5, 0.36, and 0.36 per cent for wheat, oatmeal, and corn grains respectively. There are no data available which make it possible to state whether this high content of potassium is in some degree detrimental when taken regularly, as in these experiments.

It should be emphasized that we are discussing the value of the total nitrogen of the potato, only 63 per cent of which is reported as true protein, the remainder consisting of simpler nitrogenous compounds, which may not consist entirely of complexes yielding amino-acids when digested. It remains for future investigations to show whether or not the coagulable nitrogen of the potato, or the true proteins have a higher biological value than have those of the cereal grains.

It should be pointed out further that this principle also applies to our studies on the values of the proteins of the cereal grains. We employed only the entire mixture of proteins as they exist in the seeds. The results of such studies may not be the same when a *portion* of the nitrogenous compounds of the seeds are isolated and fed separately, or in agreement with results obtained by supplementing the limited protein content of the seed with a concentrated protein preparation obtained from the same seed in order to obtain higher intakes of protein from a single source. The well known supplementary relationship between proteins which yield different proportions of certain amino-acids, should serve to invalidate results obtained by such a procedure as representing true comparisons of the relative biological values of the proteins of one foodstuff as compared with those of another. The relatively low content of protein in all the cereal grains makes it impossible to compare their values except at relatively low planes of protein intake until the problem shall be solved of isolating the *total content of nitrogenous compounds* from the greater portion of the non-nitrogenous constituents of the natural foods being studied.

The extent to which records of nitrogen intake and output in the adult may be interpreted to indicate the biological values of the proteins of the diet for the support of growth, deserves

careful consideration. This type of data, under conditions designed to show the minimum amount of protein from a given source which can replace the endogenous loss, has been frequently presented by investigators, and the results have been taken to constitute a comparison of the relative biological values of the different foods employed with respect to protein. It is imperative in the light of the very high values reported for potato nitrogen in experiments where maintenance of nitrogen equilibrium in the adults was determined, as contrasted with our much lower values for this nitrogen for the support of growth, to examine carefully the problem of whether the processes of maintenance (*i.e.* repair of endogenous nitrogenous loss) are of essentially the same type as those involving growth. McCollum (7) has reported data which support the idea that even such incomplete proteins as gelatin or zein, when fed as the sole source of nitrogen, are not without some value for the purpose of replacing nitrogen lost through endogenous catabolism. It is profitable in accounting for the discrepancy between the apparent values of the nitrogen of the potato for maintenance as contrasted with growth, to consider some of the possible explanations as to the utilization of the "incomplete" proteins for partial replacement of the nitrogen lost through endogenous catabolism.

It is now well established that during fasting the content of amino-acids in the blood is kept remarkably constant (8) through the sacrifice of muscle tissue as a source of the cleavage products of protein. When an animal is fasting or taking a diet furnishing only an incomplete protein, it is still necessary that the glandular organs continue to form such special products as the hormones which must be constantly contributed to the blood stream in order to correlate the physiological activities of the several tissues of the body. In the formation of these there is a demand for special amino-acids, presumably tyrosine for the formation of adrenalin, tryptophane for the formation of the active substance of the thyroid, etc. The selective action of the glands in using up several amino-acids from the complete list which is formed by the hydrolysis of muscle proteins would leave an incomplete mixture circulating in the blood. The formation of such special products as protamine or histone in the de-

velopment of spermatozoa illustrates another type of special activity of a gland which calls for a disproportionately large amount of certain amino-acids—in this case arginine, histidine, and lysine—and should, theoretically, leave behind in the blood an incomplete series of monoamino-acids. Under such circumstances, the situation might arise in the feeding of a diet containing an incomplete protein, that the series of amino-acids absorbed from the alimentary tract should be supplemented by those cleavage products already circulating in the blood so as to make a complete mixture capable of being utilized for the replacement of a certain amount of the protein degraded through endogenous catabolism. In an analogous manner it is theoretically possible that in certain cases, where the proteins of the food are of low biological value, their quality may be improved to a slight extent through the reutilization of the unused quota of amino-acids which remain after the draft by certain glandular tissues upon the list of these circulating in the body fluids. It is possible that the protein of the potato may fulfil these conditions, and therefore actually be of decidedly greater value for maintenance as contrasted with growth. When growth takes place, this peculiar supplementary relationship between the unused quota of amino-acids just mentioned could be of but slight importance since the magnitude of the endogenous metabolism is small.

Little is known about the nature of the proteins of the different body tissues. It is not possible therefore to judge to what extent the different glandular organs and other specialized tissues in their normal functioning would require the same proportions of the several amino-acids for their nutrition, as are yielded by the muscle proteins when the latter are hydrolyzed for the maintenance of a normal supply of these in the blood. It is hardly probable that the proteins of the glandular organs, with their highly specific functions, and the proteins of the nervous tissues, should at all closely resemble each other in the proportions of the different amino-acids which they yield. The great difference in the content of glycocoll in the collagen of the inert cartilage as contrasted with the very small amount of this complex in the proteins of the muscle is an illustration in point. Even in the nourishment of the more indispensable tissues of the body

at the expense of the partially dispensable muscle, there may remain an unused quota of amino-acids which can be reutilized together with a supply of protein digestion products which are incomplete or relatively so.

It is of great importance to determine the extent to which experimental trials based on the maintenance of nitrogen equilibrium in the adult with minimal intakes of nitrogen agree with the result of studies on the relative biological values of the same proteins for growth. Failure of such comparative studies to give concordant results in any case seems to necessitate the acceptance of some such explanation as that described above.

In order to compare the biological values of proteins from different sources, it is necessary, therefore, to make such comparison on the basis of data obtained in experiments in which there is determined the lowest level of nitrogen from the several sources required to support *growth* at a definite rate. Maintenance experiments will not take the place of studies on growth.

The value of the potato with respect to its content of fat-soluble A is about the same as that of the cereal grains. Rats have been observed to grow fairly well, and produce young, and to reach the age of 10 months without the development of xerophthalmia on diets in which the sole source of this dietary essential was 80 to 90 per cent of cooked dried potato. With diets consisting of potato supplemented with casein and inorganic salts, rats have not been able to rear young. On diets derived from a cereal grain, casein, and the same salt mixture, on the other hand, young have been reared. This does not necessarily mean that the seeds are richer in the dietary factor fat-soluble A than the potato. Small differences in the values of one or more of the other factors in the diet would account for the observed differences in quality, which are slight, between cereal and potato rations. A comparison of Charts 4 and 5 shows convincingly that the content of fat-soluble A in the potato is below the optimum, since a mixture of dried potato, casein, and salts is distinctly enhanced in its dietary properties by the addition of butter fat.

Cooked dried potato is supplemented by the addition of purified protein, sodium chloride, calcium carbonate, and a fat containing fat-soluble A, so as to be satisfactory for the support of growth at about the normal rate, and to induce reproduction and the



rearing of some of the young. The mortality of the young was high, and the fertility of the adults was decidedly below the normal. Young from these rats have grown at the normal rate for about 3 months after weaning, when confined to the diet to which their parents had been restricted throughout life (Chart 5) and one has given birth to three young.

The potatoes employed in the feeding experiments described were steamed until thoroughly cooked. They were then dipped in cold water long enough to make it possible to handle them, and the skins were removed. They were immediately passed through a ricer and allowed to fall upon cheese-cloth. The latter was supported upon wire cloth frames. The riced potato was not disturbed, and was so disposed that air could circulate through it. It was dried in a current of warm air at a temperature of about 60°C., and ground.

As in all our feeding experiments conducted during the past 5 or 6 years, all the ingredients of the food mixture were ground together so finely that it was impossible for the animals to pick out any single constituent. This was fed in a special device which prevents the animals from scattering or otherwise wasting the food and permits keeping quantitative records of the food intake.

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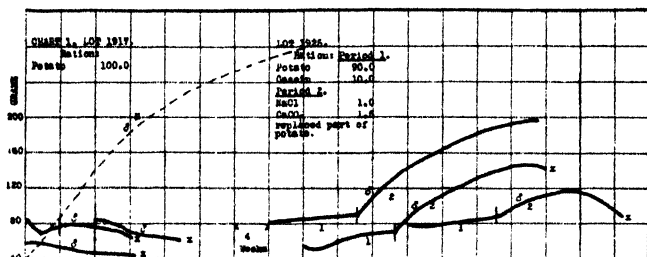


CHART 1. Lot 1917. These curves show the results of feeding young rats solely upon cooked dried potato. The potato, like the cereal grains, is deficient in three respects; viz., in a shortage of calcium, sodium, and chlorine, in the quality of its protein, and in a relative shortage of fat-soluble A. At the end of the experiment these rats were not in good condition, but there were no baldness nor skin infections. The consumption of food by the group averaged 5.2 gm. per day for each rat throughout the experiment.

Lot 1925. In Period 1 the animals were unable to grow on a diet consisting of cooked dried potato supplemented with purified protein casein. After 10 weeks suspension of growth, there was an immediate response on the addition of a simple salt mixture, which consisted of sodium chloride and calcium carbonate. The preliminary period of stunting, together with the relative shortage of fat-soluble A, forms sufficient reason for their early failure. These animals did not suffer from xerophthalmia. During the first period, these rats consumed each 4.5 gm. of food per day; during the second period 8 gm. each per day.

X = animal died.

Y = birth of young.

♂N = normal growth of male rat.

♀N = " " "female "

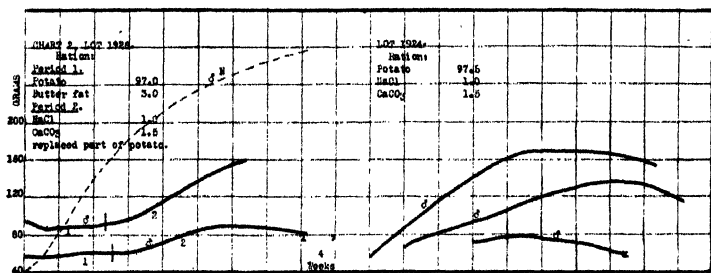


CHART 2. Lot 1926. In Period 1 the rats were unable to grow on the diet of potato and butter fat. There was a distinct response in Period 2, on the addition of the inorganic elements, sodium, chlorine, and calcium, in which the potato is poor. The response was not rapid or pronounced because of the deficiency and poor quality of the protein content of the diet. This diet contained nearly 8 per cent of protein ( $N \times 6.25$ ). With the same amount of nitrogen from the maize or wheat kernel, somewhat better growth can be secured when the same dietary deficiencies are corrected. Loss of hair, emaciation, and general poor condition were characteristic of the group. There was, however, no soreness of the ears, tail, or skin. The average consumption of food per rat was 5.6 gm. per day during the first period and 7.5 gm. per day in the second period.

Lot 1924. These records show that the nitrogen compounds of the potato are a complete source of amino-acids, but that they have no very high value for the support of growth. Without the salt additions no growth could be secured (Chart 1). It is of interest to note that none of these rats suffered an attack of xerophthalmia, indicating that the potato contains a considerable, but insufficient amount of fat-soluble A. This is further emphasized by a comparison of Charts 4 and 5. These rats (Lot 1924) were very irritable and timid. When transported from the cage to the balance for weighing they were with great difficulty prevented from escaping, since they were extremely watchful and would jump out instantly when the box was opened. In the case of the male, which failed to grow, and died early, the penis protruded to its full length persistently. We have not infrequently observed this condition in male rats suffering from faulty nutrition. The average consumption by these rats was 6.8 gm. per day per rat throughout the experiment.

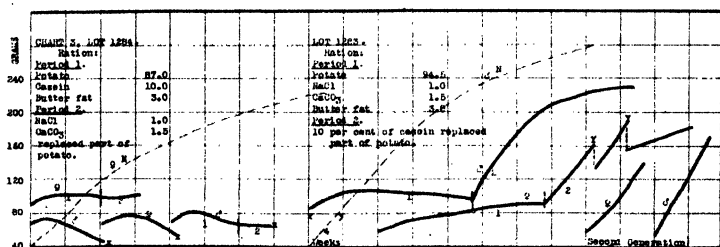


CHART 3. Lot 1284. These records show that young rats are not able to grow on a diet of potato supplemented with both a purified protein and fat-soluble A (in butter fat). The low content of calcium, sodium, and chlorine forms the first limiting factor in the dietary deficiencies of the potato. We have frequently observed that animals which have been temporarily stunted by reason of a faulty diet fail to respond with growth on the correction of the diet by suitable additions. In this group the addition of a salt mixture after 8 weeks suspension of growth did not lead to growth. Evidently the animals were injured by this diet in a much greater degree than on certain other diets which likewise fail to support growth, for in some instances there is a prompt response with growth as soon as the faults in the diet are corrected. It should be noted that these animals ate daily over a long period a much greater proportion of potato than would ever be consumed when a mixed diet is employed. Under such circumstances there may be an accumulative effect of any injurious factor in the course of time. There is much direct evidence that if there is anything injurious about the potato it is no greater than in many other of our wholesome natural foodstuffs. It is remarkable that on the addition of the salt mixture in Period 2, there was no response with growth, notwithstanding the fact that the diet in this period was identical with that of Lot 1283 (Chart 3), Period 2. We have frequently observed failure of animals to grow on correcting the errors in the diet. The average food consumption per rat per day throughout the experiment was 5 gm.

Lot 1283. In Period 1 growth took place very slowly or not at all on the diet of potato, salts, and fat-soluble A (in butter fat). In certain cases we have seen better growth on potato and salts alone (Chart 2, Lot 1924), than was secured in any of the animals in this group. In Period 2, there was a prompt response with growth in all cases on the improvement of the protein content of the diet by the addition of casein. Two females have produced three litters (10 young). One litter of two was promptly eaten by the mother. The remaining litters were successfully weaned and two of the second generation are growing normally on the diet (see curves of second generation). Considering the fact that these rats were completely stunted during a period of 19 weeks, their subsequent records indicate that the diet of Period 2 must be fairly satisfactory, notwithstanding its very high content of potato. During the first period these rats ate 6.9 gm. of food per day per rat.

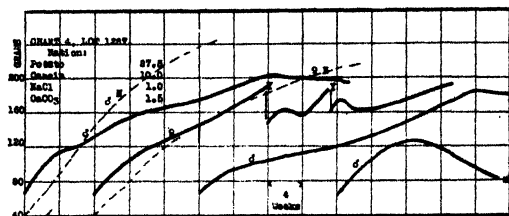


CHART 4. Lot 1287. This lot is of interest in comparison with Lot 1283, Chart 3, whose diet in Period 2 differed only in that it contained butter fat (fat-soluble A). Lot 1287 was able to grow at a fairly good rate on the diet of potato, casein, and salts, but the reproduction records fell decidedly below that of Lot 1283, even though the latter had been stunted because of unsatisfactory protein supply during 133 days before the diet was rendered complete. The two litters of young produced by one of the females were allowed to die within a short time after birth. A comparison of this chart with Chart 5 further shows that the diet of Lot 1287 was greatly enhanced by the addition of butter fat. None of these rats suffered from xerophthalmia, but were very old and rough looking at the close of the experiment. The average consumption of food was 8.26 gm. per rat per day.

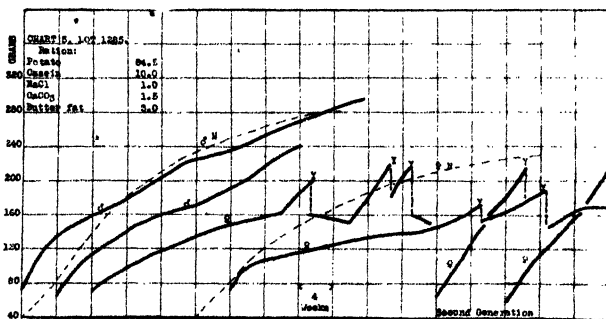


CHART 5. Lot 1285. These records show, when compared with Chart 4, that the potato does not furnish the optimum amount of fat-soluble A. The diet of Lot 1285 was like that of Chart 4, Lot 1287, except that the former contained 3 per cent of butter fat. With the diet of Lot 1285 growth was distinctly better than on the latter, and the reproduction records greatly superior. Of six litters (thirty-one young) produced by the three females, four litters (nineteen young) have been successfully weaned. Two individuals of the second generation are growing satisfactorily on the diet (see curves of second generation).

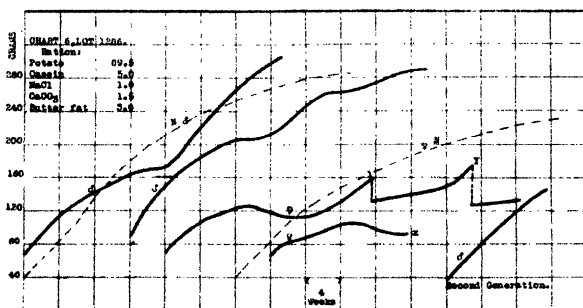


CHART 6. Lot 1286. These records are of interest in comparison with those of Chart 5, Lot 1285. The diet of Lot 1285 contained 10 per cent of casein, whereas that of Lot 1286 contained 5 per cent. There is no room for doubt that the higher level of casein serves best to promote the well-being of the animals, but the addition of 5 per cent to the proteins of the potato served to enhance greatly the value of the latter. Three young were reared from three litters (nine young). The average consumption of food was 11.1 gm. of food per day per rat.

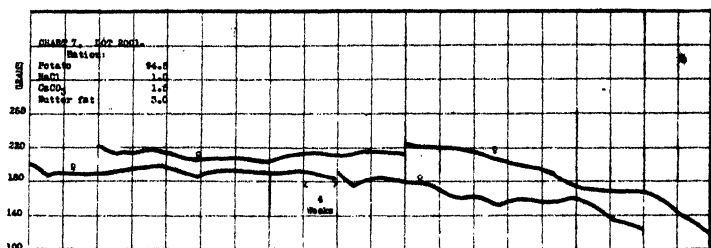


CHART 7. Lot 2001. These records show that the adult rat can take a diet 94.5 per cent of which is dry potato, and remain in apparent good health over several months. These rats were about 9 months old when they were placed on this diet, and some are still in fair condition after 9 months on this monotonous food mixture of dry cooked potato, salts, and fat-soluble A (butter fat). One female is now very emaciated, and has lost most of its hair; the skin appears normal. They are all prematurely aged in appearance although only half through the ordinary span of life of the well-fed rat. This observation harmonizes with those which we have previously made: that restricting an animal to approximately the actual minimal amounts of any dietary factor, which just cover its needs, will be followed by early signs of deterioration.

These rats were noted to be in excellent condition 9 weeks after the beginning of the experiment. Such a result suggests great caution in arriving at the conclusion that maintenance experiments in the adult even though of a somewhat prolonged character, demonstrate the satisfactoriness of a diet because no untoward results are apparent. Maintenance on 8 per cent of potato protein ( $N \times 6.27$ ) compares favorably with that obtained with a diet properly adjusted but containing only 7 per cent of wheat or oat proteins (unpublished data).

## THE DECREASED PLASMA BICARBONATE DURING ANESTHESIA AND ITS CAUSE.

### A REPORT OF PLASMA CO<sub>2</sub>, BLOOD AND URINE KETONE, AND BLOOD CATALASE ANALYSES IN OPERATIVE PATIENTS.\*

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(Received for publication, June 17, 1918.)

#### INTRODUCTION.

Caldwell and Cleveland,<sup>1</sup> Cannon,<sup>2</sup> and others have observed that anesthesia and operation are followed by a decrease in the bicarbonate content of the blood plasma. The cause of the decrease has not been demonstrated. On the basis of present knowledge, three hypothetical explanations may be proposed.

(1) Since acetone bodies are demonstrated in the urine,<sup>1, 3, 4</sup> sufficient quantities of  $\beta$ -hydroxybutyric and acetoacetic acids may be formed to decompose the bicarbonate that disappears. In this case the acetone bodies might be retained in the body, but they might also be excreted in the urine, taking with them the alkali with which they had combined in the body.

(2) Organic acids other than the acetone bodies may be formed and assist the latter in lowering the blood bicarbonate.

(3) The fall in bicarbonate may be due chiefly, not to any acids, but to the transfer of bicarbonate from plasma to body cells as a result of the increased ventilation during anesthesia. Artificially

\* Preliminary report read before the Pathological Society of Philadelphia. March 28, 1918.

<sup>1</sup> Caldwell, G. A., and Cleveland, M., *Surg., Gynec. and Obst.*, 1917, xxv, 22.

<sup>2</sup> Cannon, W. B., *J. Am. Med. Assn.*, 1918, lxx, 531.

<sup>3</sup> Reicher, K., *Z. klin. Med.*, 1908, lxxv, 235.

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increased respiration has been recently shown by Henderson and his associates<sup>5</sup> to lower not only the free carbonic acid, but also the bicarbonate of the blood, the latter shifting from blood plasma to cells when the free carbonic acid is lowered below normal limits. In this special condition the blood bicarbonate is no longer a measure of the total body bicarbonate, and a lowering of the blood bicarbonate does not indicate a decrease in the alkaline reserve of the body.

The present work was undertaken to obtain data which would give some indication as to whether one of the above factors or a combination of them causes the decrease of the bicarbonate observed during anesthesia and operation. In case the increase of acetone bodies in the blood should prove sufficient to account alone for the fall in bicarbonate, the other two factors would be demonstrated to be of, at most, minor importance.

Accordingly, quantitative estimations of the "total acetone bodies" (acetone, acetoacetic acid, and  $\beta$ -hydroxybutyric acid) of the blood were made before and after operation in 60 patients from the clinic of Dr. John B. Deaver, to whom we express our thanks for his coöperation and for the clinical data. Bicarbonate determinations on the plasma were carried out at the same time.

#### *Technique.*

About 20 cc. of blood were drawn a few hours before operation from a vein in the bend of the elbow, with due precautions to prevent stagnation. Powdered potassium oxalate was used to prevent clotting and paraffin oil to prevent escape of  $\text{CO}_2$  in the sample for  $\text{CO}_2$  estimation. For the latter, the blood was centrifuged immediately and the plasma saturated with a mixture of 5.5 per cent  $\text{CO}_2$  in air from a tank, and analyzed for  $\text{CO}_2$  with the apparatus and according to the technique of Van Slyke.<sup>6</sup> Results are expressed in cc. of  $\text{CO}_2$  at  $0^\circ$  and 760 mm. pressure per 100 cc. of plasma. For the "total acetone body" determinations, 10 cc. of whole blood were analyzed by the method of Van Slyke and Fitz.<sup>7</sup> The results express the sum of acetone, aceto-

<sup>5</sup> Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1918, xxxiii, 333, 345, 355, 365.

<sup>6</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

<sup>7</sup> Van Slyke, D. D., and Fitz, R., *J. Biol. Chem.*, 1917, xxxii, 495.

acetic acid, and  $\beta$ -hydroxybutyric acid in terms of mg. of acetone per 100 cc. of blood. The patient's clinical history was noted, the manner of taking the anesthetic, and the operation and findings were added. The duration of anesthesia was counted in minutes from the administration of the first drop until the gauze was removed, the open drop method of anesthesia being used. A second sample of blood was then obtained and subjected to the same analyses. Observations of the postoperative condition were the final steps. Ether was the anesthetic in all but one case, in which nitrous oxide was used.

Quantitative determinations of the ketone excretion in the urine were performed in twenty-eight cases by the technique of Van Slyke.<sup>8</sup> The quantities expressed are the amounts excreted in 24 hours after the last voiding, which was performed immediately before operation. They represent, therefore, the total amount excreted 24 hours after the beginning of anesthesia and operation.

The percentage of excretion of phenolsulfonephthalein is appended in a few cases.

Determinations of the catalase content of the blood were performed before and after operation in twenty-seven cases for purposes to be discussed below. The technique was the well known one of adding a standard amount of blood to a standard amount of hydrogen peroxide and collecting the evolved oxygen in an inverted burette during a standard time interval. 0.5 cc. of blood were used, 50 cc. of hydrogen peroxide plus about 200 cc. of water; the time was 10 minutes. The bottle containing the blood and hydrogen peroxide was placed in a water bath at 37° during the collection of the oxygen. Results are expressed in cc. of oxygen at 0° and 760 mm. pressure.

Calculations of the theoretical decrease in CO<sub>2</sub> corresponding to the increase in ketones, calculated as acetone in M-concentration, are in the table.

A very definite increase in the amounts of ketone bodies in the blood occurred during anesthesia and operation. (For normal figures, Van Slyke and Fitz<sup>7</sup> have given up to 1 to 2 mg. per 100 cc. Many of our patients were young men in the best of health except for a hernia or varicocele, and some were operated

<sup>8</sup> Van Slyke, *J. Biol. Chem.*, 1917, xxxii, 455.

TABLE

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms.*
1	35	F.	Stout.	4 yrs.	Frequency of urination; swelling of abdomen. Myoma uteri, supravaginal amp. Left salpingo-oophorectomy. Appendectomy.	Nausea no vomiting.
2	31	M.	"	?	Left inguinal hernia and varicocele. Radical operation.	None.
3	28	F.	"	3-4 mos.	Menorrhagia. Dilatation and curettage.	"
4	30	"	Moderate.	Several yrs.	Pain in epigastrium; belching of gas; attacks with jaundice. Cholecystectomy.	Much gas.
5	28	M.	"	—	Left inguinal hernia. Radical operation.	Vomited once, much belching.
6	16	F.	Thin.	2 yrs.	Pain in lower right abdomen; vomiting. Tuberculosis of cecum; resection and ileocolostomy.	Moderate shock. Gradual decline. Died in 3 wks.
7	41	M.	Small.	—	Right inguinal hernia. Radical operation, appendectomy.	None.
8	28	F.	Muscular.	3 yrs.	Swelling of neck 3 yrs.; slight dyspnea. Partial thyroidectomy.	"
9	54	M.	Thin.	1 yr.	Dysuria. Prostatectomy.	Slight shock. Vomited several times.
10	28	"	Muscular.	9 days.	Severe attack, "frozen." Appendectomy with drainage.	None.
11	70	F.	Thin.	3 mos.	Carcinoma of tongue. Resection of half of tongue.	"
12	49	"	Very small.	4 yrs.	Fibroma of wrist. Excision.	"
13	26	M.	Thin.	22 days.	Lobar pneumonia. Empyema on 22nd day.	" Very slow convalescence.

\*Except where noted there was no shock and the convalescence was rapid.

Anesthesia.		CO <sub>2</sub>		Ketones.		Differences.		Theoretical decrease in CO <sub>2</sub> .	Catalase.		Urine	
Respiration.	Duration.	Before.	After.	Before.	After.	CO <sub>2</sub>	Ketones.		Before.	After.	Acetone, after	Phenolphthalein, before
	min.	cc.	cc.	mg.	mg.	cc.	mg.	cc.	cc.	cc.	gm.	per cent
Much mucous; deep snoring; oxygen given.	Gas 1	73.3	59.9	37.7	45.5	13.4	7.3	2.8				
	Ether 50											
Smooth.	" 35	72.9	61.4	3.8	9.8	11.5	6.0	2.3	186	373	0.267	
"	" 20	74.6	59.8	18.7	30.6	14.8	11.9	4.5				
Very smooth.	" 37	74.0	42.8	18.6	35.8	31.2	17.2	6.6				
Smooth.	" 45	74.3	67.1	6.0	35.3	7.2	29.3	11.3			1.306	
"	" 50	75.8	48.7	19.8	49.0	27.1	29.2	11.2	185	325		
"	" 65	80.5	61.8	10.5	21.8	18.7	11.3	4.3			0.208	
"	" 30	56.5	47.3	6.8	11.4	9.2	4.6	1.7				
"	" 36	81.5	62.6	1.3	10.2	18.9	8.9	3.4	409	372		
"	" 48	81.5	65.5	8.5	26.9	16.0	18.4	7.1	419	325	1.214	
Smooth except for tongue difficulty.	" 39	75.8	65.3	4.1	22.7	10.5	18.6	7.1	440	334	1.371	
Smooth.	" 18	78.0	66.4	6.0	11.2	12.2	5.2	2.0				
Struggling.	Gas 14	86.2	62.7	4.8	17.9	13.5	13.1	5.0	255	231	0.840	

TABLE I—

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms.*
14	52	F.	Stout.	6 yrs.	Pain in epigastrium with vomiting and jaundice. Several attacks a year ago. Cholecystectomy; appendectomy.	Some bleeding from incision. Apoplexy and death on 25th day.
15	54	M.	Thin.	8 "	Upper right abdominal pain 2-3 hrs. after meals. Much indigestion. Duodenal ulcer. Excision of ulcer and posterior gastroenterostomy.	None.
16	25	"	Muscular.	Many yrs.	Left varicocele. Radical operation.	"
17	41	"	Moderate.	"	Hemorrhoids and fissure in anus. Radical operation.	"
18	31	"	"	5 yrs.	Pain in epigastrium 1-2 hrs. after meals. Same in right iliac fossa. Appendectomy; upper abdomen explored.	Vomited; much belching.
19	56	"	Fair.	2 yrs.	Pain and bleeding. Hemorrhoids; fistula in anus.	None.
20	42	"	Muscular.	1 mo.	Pain in epigastrium; indigestion. Appendectomy, chronic appendicitis.	"
21	21	"	Moderate.	Several yrs.	Varicocele. Radical operation.	"
22	36	"	Thin.	2 mos.	Inguinal hernia. Radical operation.	"
23	40	"	Stout.	Indefinite.	Indigestion and severe upper abdominal pain. Cholecystectomy and appendectomy.	"
24	39	F.	Thin.	1 yr.	Pain in back and lower abdomen. Retroflexed uterus suspended. Appendectomy.	"
25	31	M.	Stout.	3 wks.	Pain in right iliac fossa. Appendectomy.	" Normal convalescence.

\*Except where noted there was no shock and the convalescence was rapid.

*Continued.*

Anesthesia.		CO <sub>2</sub>		Ketones.		Differences.		Theoretical de- crease in CO <sub>2</sub> .	Cata- lase.		Urine	
Respiration.	Duration.	Before.	After.	Before.	After.	CO <sub>2</sub>	Ketones.		Before.	After.	Acetone, after.	Phenol- piridin, before.
	min.	cc.	cc.	mg.	mg.	cc.	mg.	cc.	cc.	cc.	gm.	per cent
Cyanosed part of time; oxy- gen given.	Ether 97	78.7	62.7	13.7	24.8	16.0	11.1	4.2	349	222		
Poor; much oxygen given.	" 88	76.8	61.4	21.1	32.5	15.4	11.4	4.3	473	359	1.278	
Smooth.	" 35	84.4	57.6	15.4	31.9	26.8	16.5	6.3	466	475	1.621	
"	" 26	79.6	74.0	5.9	28.4	5.6	22.5	8.6			0.337	
Quiet; oxygen given.	" 58	79.6	58.9	11.0	12.6	20.7	1.6	0.6				
Smooth.	" 30	68.3	68.3	22.5	35.3	0	12.8	4.9			0.438	
"	" 36	79.6	61.6	1.8	19.2	18.0	17.4	6.7			0.432	70
"	" 14	78.7	58.9	4.7	13.4	19.8	8.7	3.3				
"	" 24	78.6	64.3	26.2	36.1	14.3	9.9	3.8	366	233	0.814	
"	" 60	76.8	64.3	49.2	112.4	12.5	63.2	24.3	371	396	1.968	85
"	" 32	73.0	59.1	45.8	52.0	13.9	6.2	2.3	402	440	11.0	90
"	" 19	74.0	51.3	46.4	301.8	22.7	255.4	98.5	491	402		50

TABLE I—

Case No.	Age.	Sex.	Body conditions.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms.*
26	22	F.	Thin	2 mos.	Pain in lower abdomen; menorrhagia; leucorrhea. Supravaginal hysterectomy. Double salpingo-oophorectomy.	None.
27	19	"	"	1 yr.	Presence of goiter. Partial thyroidectomy.	Marked shock. Very rapid pulse; toxic 3-4 days.
28	42	M.	Moderate.	4 mos.	Vomiting. Pyloric obstruction, posterior gastroenterostomy.	None.
29	20	F.	Thin.	5 "	Intervals of pain in right iliac fossa. Appendectomy.	Vomited.
30	26	M.	Muscular.	2 yrs.	Pain in epigastrium, relieved by food. Ulcer, duodenum excised. Postgastroenterostomy, appendectomy.	"
31	41	F.	Thin.	12 yrs.	Pain in epigastrium, relieved by eating; gastric ulcer; circular resection. Appendectomy.	None.
32	52	"	Moderate.	14 mos.	Tumor right breast; carcinoma. Radical amputation.	"
33	50	M.	"	1 yr.	Indigestion and pain in right upper and lower abdomen. Exploratory operation. Appendectomy.	"
34	41	F.	"	2 yrs.	Small tumor right breast; adenofibroma. Excision.	"
35	58	M.	Muscular.		Right inguinal hernia. Radical operation.	"
36	29	F.	Moderate.	2 yrs.	Pain and swelling in lower abdomen. Myoma; hysterectomy. Double salpingo-oophorectomy. Appendectomy.	"
37	30	M.	Thin.	3-4 yrs.	Pain in epigastrium 3-4 hrs. after eating. Duodenal ulcer excised postgastroenterostomy; appendectomy.	"
38	18	"	"	1 yr.	Mild pain in right iliac fossa. Appendectomy.	"

\*Except where noted there was no shock and the convalescence was rapid.

Continued.

Anesthesia.		CO <sub>2</sub>		Ketones.		Differences.		Theoretical decrease in CO <sub>2</sub> .	Catalase.		Urine.	
Respiration.	Duration.	Before.	After.	Before.	After.	CO <sub>2</sub>	Ketones.		Before.	After.	Acetone, after.	Phenolphthalein, before.
	min.	cc.	cc.	mg.	mg.	cc.	mg.	cc.	cc.	cc.	gm.	per cent
Smooth.	Ether 53	74.9	47.2	10.5	50.3	27.7	39.8	15.3	415	306	2.264	
"	" 54	42.0	28.1	103.7	185.6	13.9	81.9	31.6				
"	" 90	63.8	47.1	18.7	30.4	16.7	11.7	4.5				
"	" 77	59.7	54.9	32.7	39.9	4.8	7.2	2.7				
"	" 102	81.3	65.1	10.2	30.6	16.2	20.4	7.8				
"	" 131	60.7	44.3	30.7	126.7	16.4	96.0	37.0				
"	" 50	74.9	52.4	5.7	9.2	22.5	3.5	1.3				
"	" 40	79.9	63.2	21.5	25.1	11.7	3.6	1.3			1.276	45
"	" 22	73.9	51.4	3.8	5.8	22.5	2.0	0.7	348	231	0.699	
"	" 30	80.2	56.5	28.5	49.9	23.7	21.4	8.2	402	167	1.468	70
"	" 50	70.2	50.8	9.0	38.6	19.4	29.6	11.4				
Rough cyanosis; oxygen given.	" 67	80.5	65.3	6.2	18.7	15.2	12.5	4.8	426	352	0.070	
Smooth.	" 30	70.2	57.0	40.5	76.7	13.2	36.2	13.9				



TABLE 1—

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms.*
39	19	M.	Muscular.	Several yrs.	Varicocele. Radical operation.	None.
40	26	"	Moderate.	2 days.	Pain in right iliac fossa; nausea, vomiting. Appendix abscess; appendectomy and drainage.	"
41	42	F.	Stout.	2 yrs.	Pain in lower abdomen; metrorrhagia. Hysterotomy and curettage; retained membranes removed; appendectomy.	"
42	42	"	Moderate.	3 mos.	Swelling in vagina; cyst excision.	"
43	49	"	Thin.	6 days.	Pain over abdomen; vomiting. Appendix abscess; appendectomy and drainage.	"
44	36	"	"	3 mos.	Pain over lower abdomen; tubal abscess. Double salpingo-oophorectomy. Appendectomy and drainage.	Moderate shock; vomiting; restless. Slow convalescence.
45	33	M.	Muscular.	8 "	Pain in upper abdomen; anemia. Splenectomy; appendectomy.	Moderate shock. Slow convalescence.
46	36	"	"	3 days.	Abdominal pain, localizing to right iliac fossa. Appendectomy and drainage.	None.
47	21	F.	Thin.	2 wks.	Pain in lower abdomen; vomiting. Appendectomy and drainage.	"
48	44	M.	Stout.	8 yrs.	Pain in right iliac fossa. Indigestion. Chronic appendicitis; appendectomy.	"
49	25	"	Moderate.	2 days.	General abdominal pain, localizing to right iliac fossa. Appendectomy and drainage.	"
50	38	F.	Stout.	2 yrs.	Pain in right loin radiating to groin; hematuria. Ureteral calculus removed.	Moderate shock.

\* Except where noted there was no shock and the convalescence was rapid.

*Continued.*

Anesthesia.		CO <sub>2</sub>		Ketones.		Differences.		Theoretical decrease in CO <sub>2</sub> .	Catalase.		Urine.	
Respiration.	Duration.	Before.	After.	Before.	After.	CO <sub>2</sub>	Ketones.		Before.	After.	Acetone, after.	Phenolphthalein, before.
	min.	cc.	cc.	mg.	mg.	cc.	mg.	cc.	cc.	cc.	gm.	per cent
Smooth.	Ether 25	75.8	67.3	54.8	60.0	8.5	5.2	2.0	463	457	0.344	
"	" 45	83.4	69.2	4.4	19.6	14.2	15.2	5.8			0.968	
"	" 52	77.7	47.5	5.7	26.1	30.2	20.4	7.8	262	184		
"	" 55	75.8	66.2	10.1	20.4	9.6	10.3	3.9				
"	" 70	67.2	55.7	0.6	19.5	11.5	18.9	7.2				
"	" 80	86.2	62.2	63.3	203.5	24.0	140.2	54.1				
"	" 50	74.0	57.8	2.5	28.4	16.2	25.9	9.9	395	268	0.592	65
"	" 48	81.3	56.7	7.1	11.6	24.6	4.5	1.7				
"	" 40	69.3	65.1	10.1	22.3	4.2	12.2	4.7				
"	" 30	83.4	65.3	11.4	35.6	18.1	24.2	9.3	339	225	1.196	55
"	" 30	83.4	62.4	2.0	5.6	21.0	3.6	1.3	395	381	0.326	
"	* " 75	74.0	62.4	5.0	9.7	11.6	4.7	1.8				

TABLE I—

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms.*
51	33	F.	Thin.	8 yrs.	Backache; menorrhagia. Myoma; hysterectomy.	None
52	23	"	"	1 yr.	Right femoral hernia. Herniorrhaphy.	"
53	33	M.	Moderate.	5 mos.	Pain in upper abdomen; vomiting. Duodenal ulcer excised. Postgastroenterostomy. Appendectomy.	"
54	44	"	Muscular.	2 yrs.	Hemorrhoids. Clamp and cautery.	"
55	21	"	Thin.	2 "	Soft inguinal hernia. Herniorrhaphy.	"
56	40	F.	Moderate.	6 wks.	Lower abdominal pain; leucorrhea. Chronic tube ovarian disease. Double salpingo-oophorectomy. Appendectomy.	Moderate shock
57	47	"	"	5 yrs.	Epigastric pain; vomiting. Duodenal ulcer; pylorectomy. Postgastroenterostomy. Appendectomy.	None.
58	33	M.	"	2 "	Mild recurring pain in right iliac fossa. Chronic appendicitis; appendectomy.	"
59	45	"	Thin.	4 mos.	Dysuria; pyuria. Tuberculous kidney; nephrectomy.	"
60	22	"	"	2 wks.	Pain in right iliac fossa. Chronic appendicitis; appendectomy.	"

\*Except where noted there was no shock and the convalescence was rapid.

*Concluded.*

Anesthesia.		CO <sub>2</sub>		Ketones.		Differences.		Theoretical decrease in CO <sub>2</sub> .	Catalase.		Urine.	
Respiration.	Duration.	Before.	After.	Before.	After.	CO <sub>2</sub>	Ketones.		Before.	After.	Acetone, after	Phenol-phthalein, before
	min.	cc.	cc.	mg.	mg.	cc.	mg.	cc.	cc.	cc.	g m.	per cent
Smooth.	Ether 54	70.0	56.7	51.2	89.0	13.3	37.8	14.5	419	373	1.450	70
"	" 35	78.7	65.3	9.2	17.6	13.4	8.4	3.2			6.908	
"	" 90	64.8	43.8	12.8	28.1	21.0	15.3	5.9				
"	" 20	81.5	68.4	6.5	13.9	13.1	7.4	2.8	279	325		
"	" 62	81.5	71.0	13.6	29.8	10.5	16.2	6.2	349	323		
"	" 54	56.6	47.0	4.7	51.2	9.6	46.5	17.9				
"	" 73	80.5	66.1	16.7	30.4	14.4	13.7	5.2			0.688	85
"	" 31	78.4	55.4	20.8	46.6	23.0	25.8	9.9				
Cyanosed throughout; not smooth.	Gas 3	68.1	52.8	0.6	52.8	15.3	52.2	20.1	389	325		40
Smooth.	Ether 62											
	" 60	73.0	70.2	56.3	63.6	2.8	7.3	2.8	440	316	0.137	

to enable them to enlist in some branch of military service. Their average ketone content before operation was 17 mg. per 100 cc.

Only 10 of 60 patients or 16 per cent showed a diminution of the CO<sub>2</sub> capacity to below 50 cc., and in only 1 case did it fall below 40 cc. above which Stillman, Van Slyke, Cullen, and Fitz<sup>9</sup> found no marked symptoms of acidosis *per se* in diabetes. Those of our patients who showed a CO<sub>2</sub> capacity even below 50 cc. however, showed a much higher percentage of postoperative symptoms, such as rapid pulse, restlessness, gas pains, etc., than the others.

Especial attention is drawn to Case 27, who had noticed a goiter for about a year, but had no other symptoms than the presence of a small swelling. On admission, the patient was somewhat nervous, but her pulse remained around 90 per minute until she was about to be carried to the operating room, when it reached 140, and she became obviously very much unsettled. Anesthesia lasted 54 minutes and was very smooth. Her pulse was then 170, but of good volume. For 3 days it remained from 120 to 160 and she appeared very toxic; on the 4th day she recovered from her toxic symptoms very rapidly, so that by the 5th day the prognosis was undoubtedly favorable, from having been extremely grave for 3 days and doubtful on the 4th. Her CO<sub>2</sub> capacity before operation, 42 cc., indicated an already existing acidosis and the fall of 13.9 cc., bringing her capacity to 28.1 cc., showed that her alkali was brought to a level which was indeed critical. Her ketones before operation were at the very high level of 103.7 mg., with an increase of 81.9 mg.

Women, according to the observers mentioned, excrete larger amounts of ketone bodies in their urine after operation than men. Their results are only very approximate on account of the technique used.<sup>10</sup> The amounts in the blood and urine in this series are therefore interesting. Twenty-six of these patients were women and their average total acetone bodies in the blood was 21.1 mg., with an increase to 47.2 mg. The average content of the men was 16.4 mg. with an increase to 40.3 mg. The average fall in CO<sub>2</sub> in women was 16 cc.; in men, 18.5 cc. The data of

<sup>9</sup> Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, 1917, xxx, 405.

<sup>10</sup> Qualitative determinations of the urine by the sodium-nitroprusside and the ferric chloride methods for ketones often yielded negative results, when quantitative estimations by the Van Slyke method yielded an appreciable precipitate.

excretion were obtained in only seven women; their average was 3.4 gm. in 24 hours. In twenty-one men, the average was 0.814 gm.

A number of patients with peritonitis from various causes were fasted for 1 to 3 days before operation. These might be expected to have a higher blood ketone on account of deprivation of food alone, but their average was 6.6 mg. The increase after anesthesia and operation, however, averaged 18.4 mg., the largest of any group. The fall in the  $\text{CO}_2$  capacity averaged 17.6 cc.

Those who were given the anesthetic for less than 60 minutes showed an average fall of  $\text{CO}_2$  capacity of 17.0 cc. and a rise of ketones of 23.4 mg. Those who received the anesthetic over 60 minutes showed a fall of  $\text{CO}_2$  capacity of 14.4 cc., and a rise in ketones of 21.7 mg. Those under 30 minutes showed a fall in  $\text{CO}_2$  capacity of 13.5 cc., and an increase in ketones of 13.2 mg.

From the standpoint of postoperative symptomatology, most of the patients recovered promptly and with few troubles. A few, however, were nauseated and vomited several times, and some were annoyed by gaseous distention. Their average fall in  $\text{CO}_2$  capacity was 16.7 cc.; the average rise in ketones, 27.3 mg.

Other patients showed some postoperative restlessness with rapid pulse, though none in the series was actually shocked. The fall in  $\text{CO}_2$  capacity of these was 17.5 cc., and the average increase in ketones was 38 mg., corresponding in molecular equivalent to a  $\text{CO}_2$  change of 15 cc.

In the entire series of cases the average increase in total acetone bodies calculated as acetone was 25 mg. per 100 cc., or 0.0043 M in concentration, corresponding to a change in bicarbonate  $\text{CO}_2$  of 9.6 cc. in 100 cc. of blood. The average observed fall in bicarbonate  $\text{CO}_2$  was 15.9 cc. These figures indicate that the acetone bodies which appear in the blood are sufficient to account on the average for 60 per cent of the observed fall in bicarbonate. They are, therefore, not the only factor, but as a rule, they are an important one in reducing the plasma bicarbonate during anesthesia and operation.

When cases are considered individually, the results show inconsistencies. In 13 of the 60 cases, the increase in acetone bodies equals or exceeds in molecular concentration the fall in bicarbonate. Case 25, representing the extreme in this respect,



## THE INFLUENCE OF CERTAIN SALTS ON ENZYME ACTION.

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(Received for publication, August 14, 1918.)

In the course of some bacteriological studies by Winslow and Falk (1) on salt action the question arose as to whether the antagonistic effects of salts on the viability of organisms which were observed were to be attributed to changes in the permeability of the organisms to the fluid in which they were suspended, following the conclusions reached by Loeb from his experiments on marine forms, or whether the explanation might not lie with disturbances in the metabolism of the organisms effected by the salts through action on the enzymes. Specifically, the question was: Can an antagonistic action of salts on enzymes be demonstrated? This question was deemed worthy of careful study, especially when it was realized that there is no inherent confliction between the two methods of explanation of the salt effects. The difference is only in the mechanism of the reaction. Inasmuch as enzymes are of colloidal nature and, further, Czapek's (2) modification of Overton's conception of the nature of a plasmatic membrane involves the existence of a colloidal phase, it is at once evident that an experimental demonstration of antagonism on enzyme action might be interpreted to mean that the salts are producing here the same effect on the colloidal systems containing the enzyme which they produce on the membrane in cell permeability changes.

The studies of Kastle and Loevenhart (3) and other workers have demonstrated the presence of lipase in all tissues examined by them containing fat. There does not seem to be any apparent objection to the conception that if the plasmatic membrane of a cell contains a lipoid phase it may also contain a lipoclastic enzyme and that the salts may produce such effects through the enzyme



on the lipid phase as will result in changes in permeability. This suggested possibility is more or less in agreement with the well known fact that the lysis of cells and the diffusion of the constituents into the surrounding medium usually take place very rapidly after death. The explanation may be that it is due to the presence in the peripheral portions of the cell of a lipase which hydrolyzes the lipid constituents of the plasmatic membrane.<sup>1</sup>

The first studies on the effects of salts on enzymes *in vitro* were undertaken on diastases and proteases. Besides the effects which have been noted by other workers (5), no significant results were obtained. It is recognized, however, that the negative results may be more or less attributable to the cursory fashion in which these experiments were carried out. Further studies on these enzymes are planned for the future. The work on lipases was more successful, and will therefore be reported here in detail.

#### HISTORICAL.

Since the early work of Green (6) and Sigmund on vegetable lipases there have been numerous contributions to the study of the fat-splitting enzymes. Many workers have made use of lipase reactions to study the effects of a variety of extraneous substances and physical conditions upon enzyme action (3, 7-10). The conflicting results obtained by various workers on the effects of inorganic salts on lipase can frequently be explained by differences in the experimental conditions and more often by differences in the concentrations of the salts employed. This latter factor is one of very great importance. Both Kanitz (11) and Pottevin (12) found that calcium ions increase the activity of lipase. Pekelharing (13) considered the increase in action of pancreatic lipase on olive oil effected by the halides of alkali and alkali earth metals due to the formation of insoluble soaps,

<sup>1</sup> The term plasmatic membrane is used in the sense in which it has been employed by Czapek and does not necessarily predicate the existence of an actual membrane. The objection of Fischer (4) to the use of the term "plasma membrane" and the suggested substitution of the conception of "colloid effects" cannot be considered as more than a verbal quibble. There is no easily apparent reason why effects on a "plasma membrane" may not be "colloid effects."

resulting in the removal of the oleic acid from the reaction mixture. This point is based on the belief that the end-products of enzyme reactions *in vitro* inhibit the action of the enzyme, probably by combination with it, or, as is more likely in this case, by the effect of the increasing concentration of hydrogen ions. Loevenhart and Peirce (14) found that hydrofluoric acid and sodium fluoride inhibit the action of lipase when they are present in concentrations of about 1 part in 500. In very much lower concentrations sodium fluoride stimulates slightly. The halides and nitrates of Na and K, and the chlorides of Ca, Ba, Mn, Cd, etc., were found to be slightly inhibitive.  $\text{Na}_2\text{HPO}_4$  and  $\text{K}_2\text{CrO}_4$ , on the other hand, showed marked stimulative effects. Gerber (15) also reports that  $\text{CaCl}_2$  and neutral salts of alkali metals inhibit the action of lipase of latex. In a very comprehensive report Terroine (16) records having found that the concentration of the salts studied (the halides of sodium) determined the nature of their influence on the action of pancreatic lipase on olive oil. In very dilute solution they stimulated the action; in more concentrated solution they inhibited. This is in agreement with the work of Loevenhart and Peirce (14) on the effect of NaF on lipase action. Using somewhat different experimental conditions from those of Terroine, Hamsik (17) found only inhibiting effects produced by the chlorides of sodium and calcium. The announcement of Mathews (18) as a general law that the toxic action of cations as such and of anions as such is an inverse function of, and is determined by their solution tensions has failed to be confirmed by Pond (19, 20). Finally, Falk (21) reported having found that the halides and nitrates of univalent metals all inhibited the action of castor bean lipase on ethyl butyrate, the extent of inhibition varying directly with the concentration of the salt. No stimulation of the enzyme was observed, perhaps because low concentrations of the salts were not used. Slight stimulation was obtained by dilute solutions of salts containing bivalent cations. In more concentrated solutions they showed inhibitory action.

Rosenheim and Shaw-Mackenzie (22) found that alcohol, sodium oleate, sodium cholate, sodium glycocholate, saponin, and digitonin stimulated lipoclastic action of pancreatic extract. They also made the interesting observation that: "The zymolyte

exerted a protective action on the enzyme, and this to such a degree that even the most energetic accelerator, sodium cholalate, had no action when added to a mixture of olive oil emulsion and enzyme, but in most cases more than doubled the lipolysis when added to the enzyme first. This behavior may be considered as an indirect confirmation of the view that a combination of the enzyme with its zymolyte precedes enzyme action, and further that the accelerator acts directly on the enzyme." These authors also demonstrated that serum stimulates lipase action. Further studies on the effect of organic compounds on lipoclastic reactions were made by Falk (23). He found that methyl and ethyl alcohols and acetone inhibited the hydrolysis of ethyl butyrate by vegetable lipase, the degree of inhibition varying directly with the concentration of the compound added. Glycerol and glucose showed practically no inhibitory action.

#### *Technique.*

In these experiments the purest olive oil obtainable was used as the substrate in testing the lipoclastic power of the active enzyme preparations. Three such preparations were employed in this work; the "lipolytic pancreatic extract" prepared and kindly furnished by the Fairchild Brothers and Foster, the "steapsin" of the Digestive Ferments Co., and Merck's "pancreatin." All of these were found to contain a powerful lipase.

Olive oil, as it ordinarily appears on the market for commercial and medicinal purposes, is slightly acidic in reaction because of the presence of free fatty acids. It is essentially made up of glycerol trioleate. The action of lipase on the oil is almost entirely a hydrolysis of this ester with the formation of glycerol and free fatty acid—oleic acid. When the enzyme is first added to the substrate the hydrolysis effected is very rapid, especially at the optimum incubation temperature, about 40°C. As the reaction proceeds and the amount of free fatty acid and glycerol increases the rate of splitting of the ester decreases until at the end of about 48 hours, when the reaction has reached a state of equilibrium and at which time further production of free acid can hardly be detected. This slowing down of the rate of reaction is not due to the presence of the glycerol, but is probably due to the increasing H ion concentration of the mixture.

The initial acidity of the olive oil used was equivalent to 1.15-1.25 cc. of 0.2 N NaOH per 10 cc. of oil. The NaCl and  $\text{CaCl}_2$  used in these experiments were of the very highest purity, having been very carefully purified and recrystallized. Both of these salts are insoluble in the oil.

In each experiment 9 gm. of the oil and 0.2 gm. of the dry enzyme<sup>2</sup> were used in each reaction mixture. Except when otherwise stated in the protocols of the experiments, the amount of salt added was that, which if added to 9 gm. of water, would make up a solution isotonic with a 0.85 per cent solution of sodium chloride (physiological). The actual amounts used were 0.0765 gm. of NaCl and 0.1449 gm. of  $\text{CaCl}_2$ .

In preparing a reagent mixture, the oil and salt were mixed first in a clean, dry bottle or beaker and, after being warmed to the incubation temperature, the enzyme was added. The mixture was then carefully stirred with a clean glass rod until it had assumed a homogeneous appearance. The material adhering to the rod and the sides of the container was washed down with some pure toluene (Merek). This was added because its inhibitory action on the enzyme is negligible, whereas its restrictive action on the development of contaminating organisms is sufficient to avoid any errors due to their presence. Throughout all the work sterile conditions were obtained and sterile reagents and apparatus used whenever possible. The mixtures were then incubated for a stated period of minutes or hours. At the end of the incubation period the titrations were made of the amount of free acid present. These titrations were made with nearly 0.2 N NaOH, using phenolphthalein as an indicator, the fatty acids having been extracted by the addition of about 50 cc. of 95 per cent alcohol made neutral against phenolphthalein. All titrations were made accurately to 0.05 cc. of alkali. Suitable control mixtures were always used. Corrections on the titration figures for the normality of the alkali were made so that the results could be expressed in terms of exactly 0.2 N NaOH.<sup>3</sup>

<sup>2</sup> Two of the three enzyme preparations were tested for moisture content and were found to contain between 4 and 7 per cent for the individual powers.

<sup>3</sup> At the beginning of this study the accuracy of this technique was checked by titrating mixtures containing known amounts of acid against phenolphthalein, methyl red, methyl orange, and bromothymol blue. The method was found satisfactory.

Usually the experiments were done in duplicate mixtures 37°C. was the incubation temperature in all the experiments.

#### EXPERIMENTAL.

##### I.

*Experiments I and II.*—The object of these experiments was to detect the influence of NaCl and  $\text{CaCl}_2$ , each acting separately, upon the hydrolysis of the fat effected by the lipase during a short incubation period, when the rate of action is at its maximum, and it was expected that if there is an effect of the salts on the *rate* of hydrolysis, such an effect would appear in these experiments. The incubation periods chosen were 30 and 120 minutes.

Because of the unhomogeneous character of the reaction mixture after standing in the incubator, and because of the difficulty involved in removing from it a portion as a representative sample, it was decided to use two separate but similar mixtures in each case when two incubation periods were used, in preference to using but one mixture and attempting to withdraw an aliquot portion at the end of each period.

The enzyme preparation used in both of these experiments was the same. All results are expressed to the nearest 0.05 cc. The fourth reagent mixture was used as a control because of the acidity of the oil and of the enzyme preparation. The heated enzyme mixture was prepared by being heated to 100°C. for 5 minutes and then cooled before being incubated. In both of these experiments (Table I) there was obtained a slight stimulation by the NaCl in the shortest incubation period—30 minutes. In the somewhat longer period, this salt had either no or a slightly inhibitive effect on the enzyme action.  $\text{CaCl}_2$  always showed an inhibitory action.

*Experiments III and IV.*—In these experiments the influence of the salts on the lipase action was studied under conditions similar to those observed in the previous experiments, with a single important modification. Long incubation periods, 36 and 42 hours, were used. In these experiments the influence is not so much on the *rate* of hydrolysis as upon the final equilibrium point of the reaction. The same enzyme was used in these two experi-

ments (Table II). Each mixture used in this pair of experiments was run in duplicate. Although the titrations of these duplicates are in several cases not as close as might be desired, still they are easily close enough to permit of the recognition of the different effects produced by the salts.

TABLE I.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Reagent mixture.			
	Oil + enzyme + NaCl.	Oil + enzyme + CaCl <sub>2</sub> .	Oil + enzyme.	Oil + heated enzyme.
Experiment I.				
<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
30	5.50	4.65	4.85	1.65
120	6.45	5.55	6.45	1.60
Experiment II.				
30	4.70	4.15	4.60	1.50
120	5.55	5.25	5.80	1.60

TABLE II.  
*0.2 N NaOH Required to Neutralize.*

Incubation period	Reagent mixture.			
	Oil + enzyme + NaCl.	Oil + enzyme + CaCl <sub>2</sub> .	Oil + enzyme.	Oil + heated enzyme.
Experiment III.				
<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
36	13.90	8.55	12.25	0.90
	15.60	6.70	12.00	0.90
Experiment IV.				
42	11.40	4.85	11.95	1.00
	10.65	3.50	10.50	1.05

Both of these experiments show that NaCl has practically no effect on the amount of free acid formed during a long incubation period. In other words, the equilibrium point is not markedly affected by NaCl. The inhibitive effect of the CaCl<sub>2</sub>, on the

other hand, is shown here even more clearly than in Experiments I and II. This salt has very materially influenced the establishment of the equilibrium, having brought on this condition very much earlier than in the mixtures lacking the salt.

*Experiment V.*—That the effects of the salts can be observed after the oil has first been emulsified can be seen from the titration figures for this experiment. The control, in this case, consisted of a pair of mixtures containing the unemulsified oil. The emulsification was effected by adding sufficient aqueous 0.2 N NaOH solution to the acidic oil to give a neutral reaction to phenolphthalein.

Examination of the titration figures (Table VII) brings out a few very important results. In the first place, it is evident that the emulsification of the zymolyte has had a marked effect on the

TABLE III.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Reagent mixture.			
	Emulsified oil + enzyme + NaCl.	Emulsified oil + enzyme + CaCl <sub>2</sub> .	Emulsified oil + enzyme.	Oil + enzyme.
Experiment V.				
min.	cc.	cc.	cc.	cc.
210	11.00	5.15	11.30	5.05
	11.00	4.80	12.15	5.55

lipoclastic action. The mean titration for the enzyme acting on the oil is 5.30 cc. of 0.2 N NaOH, and for the enzyme on the emulsified oil is 11.75 cc. Recalling that 1.25 cc. of the alkali had been added to the oil in emulsifying, the latter mean titration should be considered as equivalent to the higher figure of 11.75 + 1.25 cc., namely 13.00 cc. 0.2 N NaOH. The use of the latter figure is preferable for a strictly comparable analysis. Thus we have a difference of between about 5 and 13 cc. to titrate, the only difference in the technique and manipulation having been the emulsification of the oil in the case of the latter.

It was observed, a short while after the mixtures had been set in the incubator, that the emulsions had been "broken." This was probably due to the presence of the electrolytes, either added or those present in traces in the enzyme powder. The increase

in lipoclastic action following emulsification must therefore be ascribed to some other factor than an increase in surface of reaction (as in an emulsion). The only other possible factor which can be recognized here is the possible effect of the water added as the solvent for the NaOH used in emulsifying the oil. The presence of this water may be expected to throw the reaction more strongly in the direction indicated by the equation: glycerol trioleate + water  $\rightarrow$  glycerol + oleic acid. The marked inhibiting effect of the  $\text{CaCl}_2$  is evident from examination of the figures.

*Experiment VI.*—The object of this experiment was to determine whether the inhibiting action of  $\text{CaCl}_2$  on lipase is due to the extraction of water from the reaction medium because of the hygroscopic nature of the salt, resulting in a slowing down of the hydrolysis. The experiment consists of a parallel series of mix-

TABLE IV.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Reagent mixture.			
	Oil + enzyme.	Oil + enzyme + $\text{CaCl}_2$ .	Oil + enzyme + water.	Oil + enzyme + water + $\text{CaCl}_2$ .

Experiment VI.				
hrs	cc.	cc.	cc	cc.
10	7.90	4.35	7.10	6.30
	7.80	4.30	7.05	6.20

tures with and without the salt. One series contained besides the ingredients of each of the mixtures in the other series 10 cc. of pure, redistilled water. Table IV (Experiment VI) shows definitely that the inhibitory action of  $\text{CaCl}_2$  is not due to its hygroscopic nature and to the removal of water from the medium.

*Experiment VII.*—In this experiment a study was made of the effect of varying the amount of NaCl or of  $\text{CaCl}_2$ , each added alone to the oil and enzyme. The concentrations of the salts used varied from one-tenth the amount described above as giving an isotonic concentration to ten times this amount. Parts A and B of this experiment were done simultaneously and are therefore strictly comparable.

Tables V and VI show conclusively that with the addition of increasing amounts of dry  $\text{CaCl}_2$  there is an increase in inhibitive



effect on enzyme action, until at the addition of 10 times isotonic concentration of the salt the action is almost entirely stopped.

TABLE V.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Reagent mixture.					
	Oil + killed enzyme.	Oil + enzyme.	Oil + enzyme + CaCl <sub>2</sub> 0.0149 gm. 0.1 isotonic.	Oil + enzyme + CaCl <sub>2</sub> 0.1449 gm. 1.0 isotonic.	Oil + enzyme + CaCl <sub>2</sub> 0.7245 gm. 5 times isotonic.	Oil + enzyme + CaCl <sub>2</sub> 1.4490 gm. 10 times isotonic.
Experiment VII, A.						
hrs.	cc.	cc.	cc.	cc.	cc.	cc.
24	1.70	4.55	3.35	2.50	2.10	1.75
Increase in acidity due to lipolysis.		2.85	1.65	0.80	0.40	0.05

TABLE VI.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Reagent Mixture.					
	Oil + killed enzyme.	Oil + enzyme.	Oil + enzyme + NaCl 0.00765 gm. 0.1 isotonic.	Oil + enzyme + NaCl 0.0765 gm. 1.0 isotonic.	Oil + enzyme + NaCl 0.3825 gm. 5 times isotonic.	Oil + enzyme + NaCl 0.7650 gm. 10 times isotonic.
Experiment VII, B.						
hrs.	cc.	cc.	cc.	cc.	cc.	cc.
24	1.70	4.45	4.45	4.60	4.60	4.80
Increase in acidity due to lipolysis.		2.75	2.75	2.90	2.90	3.10

The addition of NaCl, on the other hand, seems to have effected a slight stimulation of the action.

*Experiment VIII.*—Experiment VIII was an exact duplication of Experiment VII except for the addition of 10 cc. of water to

each reagent mixture and for the use of a somewhat longer incubation period. Parts A and B are strictly comparable.

TABLE VII.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Reagent mixture.					
	Oil + killed enzyme + water.	Oil + enzyme + water.	Oil + enzyme + water + $\text{CaCl}_2$ 0.1 isotonic.	Oil + enzyme + water + $\text{CaCl}_2$ 1.0 isotonic.	Oil + enzyme + water + $\text{CaCl}_2$ 5 times isotonic.	Oil + enzyme + water + $\text{CaCl}_2$ 10 times isotonic.
Experiment VIII, A.						
hrs.	cc.	cc.	cc.	cc.	cc.	cc.
36	1.70	5.30	4.90	3.55	2.45	2.05
Increase in acidity due to lipolysis.		3.60	3.20	1.85	0.75	0.35

TABLE VIII.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Reagent mixture.					
	Oil + killed enzyme + water.	Oil + enzyme + water.	Oil + enzyme + water + $\text{NaCl}$ 0.1 isotonic.	Oil + enzyme + water + $\text{NaCl}$ 1.0 isotonic.	Oil + enzyme + water + $\text{NaCl}$ 5 times isotonic.	Oil + enzyme + water + $\text{NaCl}$ 10 times isotonic.
Experiment VIII, B.						
hrs.	cc.	cc.	cc.	cc.	cc.	cc.
36	1.70	5.10	5.10	5.40	4.25	3.70
Increase in acidity due to lipolysis.		3.40	3.40	3.70	2.55	2.00

The results of this experiment (Tables VII and VIII) indicate that the effects produced by the  $\text{CaCl}_2$  in Experiment VII cannot be ascribed to the hygroscopic action of the salt. In this experiment, in the presence of an excess of water, far greater than the

amount which the salt can take up, the inhibitory action of  $\text{CaCl}_2$  is just as evident. The effect of the  $\text{NaCl}$  observed here is somewhat different from that observed in the previous experiment. In the higher concentrations it exerted an inhibitory action. The stimulative action in the isotonic concentration is worthy of note.

The experiments presented up to this point have shown that the effect of  $\text{CaCl}_2$  on enzyme action is uniformly one of inhibition, the degree of inhibition effected being a direct function of the concentration of the salt. Even the smallest amount added, 0.1 isotonic (about  $1\frac{1}{2}$  parts per 1,000 of oil) showed marked inhibitive action. The effect of  $\text{NaCl}$  is entirely different, being slightly stimulative at times in isotonic and even higher concentrations, and but slightly inhibitive even at the highest concentrations employed. These results confirm those quoted above from the work of Loevenhart and Peirce (14) and Gerber (15).

A few experiments were made to determine whether any difference in effect on enzyme action could be detected when the salts were added to the oil and enzyme or to the enzyme. According to the results obtained by Rosenheim and Shaw-Mackenzie (22) with certain organic accelerators of enzyme action, a difference may be expected. The results have been unsatisfactory and will not be reported at this time.

## II.

The following series of experiments were made in an effort to detect a true antagonism between the action of the chlorides of calcium and sodium on lipase action.

*Experiment IX.*—In Experiment IX the object was to demonstrate salt antagonism on enzyme action by varying both the  $\text{NaCl}$  and the  $\text{CaCl}_2$ , keeping the total concentration of salts constant; namely, *isotonic*. Theoretically, the number of molecules of inorganic salts present in each mixture is the same. The ratio expressed at the head of each column in the table represents the ratio of sodium to calcium in molecules of their chlorides ( $\text{Na} \longleftrightarrow \text{Ca}$ ). The incubation period was  $2\frac{1}{2}$  hours.

Examination of the mean titration figures (Table IX) seems to show the existence of an antagonism between the action of the salts. There is the possibility, however, that the low figures in

the right hand columns were due merely to an increasing (absolute) amount of  $\text{CaCl}_2$  and regardless of the decreasing amounts of  $\text{NaCl}$ . The following experiments were planned to demonstrate antagonism, avoiding this possible source of error.

*Experiments X and XI.*—A definite amount of calcium chloride was added to each mixture except the control, and then varying amounts of sodium chloride were added. The incubation period was 3 hours. The figures at the head of the columns express the relative number of molecules of  $\text{Ca}$  and  $\text{Na}$  salts added.

TABLE IX.  
*0.2 N NaOH Required to Neutralize.*

Reagent mixture.						
Oil + enzyme +						
	NaCl 0.0765 gm. $\infty \longleftrightarrow 1$ .	NaCl 0.0757 gm. + $\text{CaCl}_2$ 0.0014 gm. $100 \longleftrightarrow 1$ .	NaCl 0.0735 gm. + $\text{CaCl}_2$ 0.0056 gm. $25 \longleftrightarrow 1$ .	NaCl 0.0639 gm. + $\text{CaCl}_2$ 0.0241 gm. $5 \longleftrightarrow 1$ .	NaCl 0.0510 gm. + $\text{CaCl}_2$ 0.0483 gm. $2 \longleftrightarrow 1$ .	$\text{CaCl}_2$ 0.1449 gm. $1 \longleftrightarrow \infty$ .

Experiment IX.

	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	5.60	*	5.55	5.50	5.40	4.90	4.70
	5.45	5.80	5.30	5.10	5.35	4.90	4.55
Mean titration.....	5.55	5.80	5.45	5.30	5.40	4.90	4.65

\* This mixture was lost in manipulating.

Table X shows that although there is considerable variation between some of the duplicate mixtures, on the whole there seems to be a gradation in the amount of hydrolysis effected by the enzyme increasing definitely with the increasing amounts of  $\text{NaCl}$  added to the constant amount of  $\text{CaCl}_2$ . This is evident in both experiments. Both also show that even the most favorable salt mixtures did not succeed in bringing the titration figures up to those of the controls. The  $\text{NaCl}$  did show an effect antagonistic to that of the  $\text{CaCl}_2$ , and this in a definite, stoichiometrical order. This antagonistic effect, it must be remembered, is evidenced upon the rate of hydrolysis when the rate is greatest.

TABLE X.

*0.2 N NaOH Required to Neutralize.*

Reagent mixture.						
Oil + enzyme.	Oil + enzyme + 0.1449 gm. CaCl <sub>2</sub>					
	<table><tr><td>1 + 0.</td><td>+ NaCl 0.00765 gm. 1 + 0.1.</td><td>+ NaCl 0.03825 gm. 1 + 0.5.</td><td>+ NaCl 0.0765 gm. 1 + 1.</td><td>+ NaCl 0.3825 gm. 1 + 5.</td><td>+ NaCl 0.7650 gm. 1 + 10.</td></tr></table>	1 + 0.	+ NaCl 0.00765 gm. 1 + 0.1.	+ NaCl 0.03825 gm. 1 + 0.5.	+ NaCl 0.0765 gm. 1 + 1.	+ NaCl 0.3825 gm. 1 + 5.
1 + 0.	+ NaCl 0.00765 gm. 1 + 0.1.	+ NaCl 0.03825 gm. 1 + 0.5.	+ NaCl 0.0765 gm. 1 + 1.	+ NaCl 0.3825 gm. 1 + 5.	+ NaCl 0.7650 gm. 1 + 10.	

Experiment X.

	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	5.60	3.95	4.00	3.30	4.25	4.55	5.30
	5.60	3.35	3.55	3.40	4.15	4.40	5.00
Mean titra- tion.....	5.60	3.65	3.80	3.35	4.20	4.50	5.15

Experiment XI.

	5.35	3.65	4.35	4.15	4.35	4.90	4.70
	5.35	4.65	3.90	4.40	4.65	4.80	5.35
Mean titra- tion.....	5.35	4.15	4.15	4.30	4.50	4.85	5.05

• TABLE XI.

*0.2 N NaOH Required to Neutralize.*

Reagent mixture						
Oil + enzyme.	Oil + enzyme + 0.1449 gm. CaCl <sub>2</sub>					
	1 + 0.	+ NaCl 0.00765 gm. 1 + 0.1.	+ NaCl 0.03825 gm. 1 + 0.5.	+ NaCl 0.0765 gm. 1 + 1.	+ NaCl 0.3825 gm. 1 + 5.	+ NaCl 0.7650 gm. 1 + 10.

Experiment XII.

	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	14.70	7.90	9.25	8.95	8.50	8.50	9.20
	14.70	7.95	9.25	9.15	8.80	9.10	9.50
Mean titra- tion.....	14.70	7.95	9.25	9.05	8.65	8.80	9.35

*Experiment XII.*—This was a repetition of the two previous experiments with the single modification that a long incubation period, 36 hours, was used (Table XI).

*Experiment XIII.*—This was an exact duplicate of Experiment XII excepting for the substitution of another enzyme preparation, one which proved to be not so powerful. The incubation period was 36 hours (Table XII).

Although two different enzymes were used in Experiments XII and XIII, good checks were obtained. They undoubtedly show an antagonism between the salts in the effects on the equilibrium point of the reaction mixtures which is reached after 36 hours incubation.

TABLE XII.  
*0.2 N NaOH Required to Neutralize.*

	Reagent mixture.						
	Oil + enzyme.	Oil + enzyme + 0.1449 gm. CaCl <sub>2</sub>					
		1 + 0.	+ NaCl 0.00765 gm. 1 + 0.1.	+ NaCl 0.03825 gm. 1 + 0.5.	+ NaCl 0.0765 gm. 1 + 1.	+ NaCl 0.3825 gm. 1 + 5.	+ NaCl 0.7650 gm. 1 + 10.
Experiment XIII.							
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	5.10	3.00	2.95	2.80	3.15	3.55	3.45
	5.20	2.90	3.00	3.10	3.05	3.65	3.40
Mean titra- tion.....	5.15	2.95	3.00	2.95	3.10	3.60	3.45

### III.

It has been suggested by Robertson that calcium chloride produces its inhibitive effect on lipase action by forming insoluble soaps. What he meant, apparently, was that insoluble soaps are precipitated about the enzyme and remove it from the reaction medium. Folin has suggested that the inhibitive effect of the salt may be attributed to the precipitation of insoluble Ca soaps with the concurrent formation of free, inhibiting hydrochloric acid. Because lipase seems to be only so slightly affected by small changes in H ion concentration and because no specific effect of HCl was known of, this interpretation seemed to be of but doubt-

ful value. Some experiments were made to test this point. The following tables present figures from representative experiments.

*Experiment XIV.*—A study was made of the effect of HCl in rather high concentration on lipase action and to compare the effect of the acid with that of calcium chloride. The concentration of HCl used was 2 times isotonic as compared to isotonic  $\text{CaCl}_2$ . The alkali necessary to titrate the acid added has been subtracted. The figures in Table XIII represent the difference.

TABLE XIII.  
*0.2 N NaOH Required to Neutralize.*

Incubation period.	Oil + enzyme.	Oil + enzyme + $\text{CaCl}_2$ .	Oil + enzyme + HCl.
Experiment XIV.			
<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
$4\frac{1}{2}$	4.85	4.40	2.25
	4.70	4.00	2.65
24	*	4.55	4.45
	6.35	4.45	4.25

\* Lost in manipulation.

TABLE XIV.  
*0.2 N NaOH Required to Neutralize.*

Experiment XV.		<i>cc.</i>
Oil + enzyme + water.....		7.40
" + " + 0.01 isotonic HCl.....		8.65
" + " + 0.05 " HCl.....		8.10
" + " + 0.10 " HCl.....		7.00
" + " + 1.00 " HCl.....		4.25

Evidently the high concentration of acid used in this experiment has markedly inhibited the lipase action. This inhibition may be ascribed to an action similar to that effected by  $\text{CaCl}_2$ , or it may be simply an effect of the enormous change in  $C_H$ . The following experiments were planned to settle this question.

*Experiment XV.*—The effect of varying amounts of HCl added to the mixtures was studied. The incubation period was 24 hours at  $37^\circ\text{C}$ . In each case the HCl added was contained in a

volume of 1 cc., and this same volume of water was therefore added to all the other mixtures (Table XIV).

*Experiment XVI.*—If the effect of  $\text{CaCl}_2$  is due to the liberation of free  $\text{HCl}$  by the formation of insoluble  $\text{Ca}$  soaps, then the effects of  $\text{CaCl}_2$  and  $\text{HCl}$  should be additive. In this experiment a study was made of this point. Isotonic concentration of the salt was used throughout. In each case the  $\text{HCl}$  was contained in 1 cc., and this volume of water was therefore added to each of the mixtures not receiving the  $\text{HCl}$ . The incubation period was 24 hours (Table XV).

These experiments demonstrate that the effect of the  $\text{HCl}$  is directly proportional to the amount added and that its effect is

TABLE XV.  
*0.2 N NaOH Required to Neutralize.*

						cc.
Oil	+	enzyme	+	water		7.05
"	+	"	+	$\text{CaCl}_2$		3.75
"	+	"	+	$\text{CaCl}_2$	+ 0.001 isotonic $\text{HCl}$	6.05
"	+	"	+	$\text{CaCl}_2$	+ 0.01 " $\text{HCl}$	5.50
"	+	"	+	$\text{CaCl}_2$	+ 0.05 " $\text{HCl}$	4.10
"	+	"	+	$\text{CaCl}_2$	+ 0.10 " $\text{HCl}$	3.20
"	+	"	+	$\text{CaCl}_2$	+ 1.00 " $\text{HCl}$	2.75

not additive with that of the  $\text{CaCl}_2$ . On the contrary, in the case of the lower concentrations of the acid its action is distinctly antagonistic to that of the  $\text{CaCl}_2$ .

#### SUMMARY.

Experiments with lipases are presented, which show that there is a fundamental difference between the nature of the influence exerted by the chloride of a monovalent metal and by that of a bivalent metal ( $\text{NaCl}$  and  $\text{CaCl}_2$ ). The sodium salt may stimulate the rate of lipase action slightly; the calcium salt always inhibits. The former shows no effect on the equilibrium point of the reaction mixture; the influence of the latter is such as to bring on this condition very much earlier than in the control lacking the salt. The inhibitory action of calcium chloride is not due to the extraction of water from the medium by the salt.



The inhibiting action of calcium chloride was found to vary directly with the concentration of the salt, even in the presence of an excess of water. The concentration of sodium chloride does not appreciably affect its influence on the enzyme action.

The addition of NaCl reduces the inhibitory effect produced by the calcium salt upon both the rate of action and upon the equilibrium point. There is a definite antagonism exerted between the two in an enzyme reaction mixture and this antagonism is of a definite, stoichiometrical order. It is probable that the effect of the sodium salt is not antagonistic in an opposite sense to that of the calcium salt, but rather that the presence of the former tends to prevent the appearance of the calcium effect. That this phenomenon will be found to be general for salts of univalent metals as against salts of bivalent metals is expected, but experimental demonstration has not yet been undertaken.

It is pointed out that the inhibitory effect of the  $\text{CaCl}_2$  is probably not attributable to the formation of insoluble Ca soaps with the concurrent formation of HCl. Although this acid, when added in large amounts, does inhibit the lipase action, its action may be attributed merely to changes in the  $C_H$  of the medium. The effect of the acid is of a different nature from that of the salt as witnessed by the fact that even the smallest concentrations of the latter always inhibit the enzyme action, whereas small amounts of the acid even stimulate it slightly and prevent, to a certain extent, the appearance of the Ca effect when the salt is also added.

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## FEEDING EXPERIMENTS WITH RAW AND BOILED CARROTS.\*

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(Received for publication, September 7, 1918.)

In the present food crisis, it will perhaps be conceded that our cheapest sources of calories and protein are the vegetable fats and cereal grains. It will also be readily conceded that these foods require to be judiciously fortified or supplemented in several respects. It has often been urged that some of the common root vegetables should be used as extensively as may be practicable, as partial substitutes for the cereals, as supplements to them in certain respects, and certainly as supplements to the vegetable oils. A knowledge of the dietetic properties of these root vegetables is then of importance, particularly so since the use of dehydrated vegetables promises to come into general vogue.

In the preparation of these dehydrated products for table use it will not necessarily be the case that the excess of water contained by the vegetable in its natural state, is restored. Thus the calorie and protein values of these cooked preparations will more nearly approach those of our staple carbohydrate foods such as cereal porridges and breads, or cooked potatoes. The "watery vegetables" can then no longer be neglected as a source of fuel or calories.

As for protein, they may be so selected as to yield large amounts of nitrogen if they can be eaten in fairly concentrated form. For it will be noted that when protein (or rather, nitrogenous) calories

\* We take pleasure in acknowledging our indebtedness to Major A. J. Carlson, under whose direction this work was begun, to Dr. Arno B. Luckhardt, under whom it has been continued, also to Dr. F. C. Koch, of the Department of Physiological Chemistry, who has shown us many favors in the way of laboratory facilities.

are compared with total calories, spinach furnishes as large a proportion of nitrogen calories as some cuts of meat; tomatoes and cabbage more than wheat; and carrots almost as high a proportion as does corn-meal (1). Quantitatively, then, the vegetables above mentioned, when in dry form, would present equivalents or substitutes for meat, wheat, and corn-meal respectively, whether considered as fuel or as nitrogenous foods. However, it is true that the question of digestibility remains to be considered, since we live not by what we eat but by what we digest; and the quality of these vegetable proteins is all important, since in the last analysis it is not proteins as a class, but various individual amino-acids, which are able to bring about growth or to maintain nitrogenous equilibrium.

With these considerations in mind, we have instituted feeding experiments with carrots, using albino rats. They take this food readily, whether it is given raw, cooked, or dried. They become accustomed to it in a few days and increase their consumption gradually until they are in many cases taking each day an amount which (calculated as weight of fresh vegetables) is equal to or greater than their own body weight.

Three litters of rats were given equal representation in each of Lots I, II, and III, in the hope of eliminating as far as possible, the influence of individual variations.

Growth curves of Lots I, II, and III (Chart I, February 5 to March 12), show that after becoming adjusted to the food, young rats may maintain body weight without loss over a period of at least 5 weeks, on a diet of chopped carrots fortified only with suitable amounts of sodium chloride, calcium phosphate, and calcium lactate. (Some animals survive much longer than this and seem able to maintain their weight on this diet for long periods.) As the curves for food consumption show, these animals took from 0.5 to 1 gm. of carrot proteins per day, and maintained body weights of 50 to 90 gm. respectively. This is equivalent to 10 gm. of protein per kilo of body weight, or about six times the amount allotted to an adult man on the basis of 120 gm. of protein for 70 kilos of body weight,—which is a high protein diet as human dietaries are usually reckoned. Similarly, as far as calories are concerned, these animals on an exclusive carrot diet

were consuming an amount of food which yielded 300 to 500 or more calories per kilo of body weight per day; whereas 50 calories per kilo per day for an adult man at moderately hard work, or 100 calories per kilo for the new born human infant, are considered liberal standards. Yet these rats were unable to make a growth upon this diet. Such facts lead us to conclude, that if we are to attempt to apply widely Rubner's laws of constant energy consumption and growth quotient (2) we must at the outset hedge about that generalization rather narrowly by defining the rate of growth to be expected and the quality as well as quantity of nutrients to be fed.

It has often been contended that since most feeding experiments with rats have been done by the use of dried and often of cooked materials, it is unreasonable to suppose that the processes of drying and ordinary cooking can prejudicially influence vitamin content or other nutritive values. Further consideration, however, makes it apparent that quantitative as well as qualitative experimentation is desirable. The fact that the animal maintains a normal growth curve and reproduction on a daily diet consisting, for example, of 10 gm. of bread and 5 gm. of heated dried milk (equivalent in bulk to 50 gm. of fresh milk), does not in itself prove that the cooking and drying have not in any way altered the nutritive value of milk. For it is conceivable (until the contrary has been proved) that the animal might have been as satisfactorily nourished on 10 gm. of bread, plus 25 gm. of fresh milk, instead of using the equivalent in dried form of twice that amount.

It is well recognized that temperatures considerably above 100°C., when applied for one or more hours, do prejudicially affect the nutritive values of some foods (3). Some investigators have concluded that ordinary cooking or even pasteurizing processes are liable to weaken or destroy the antiscorbutic or other valuable properties of milk or of vegetables, in some instances at least. We therefore considered that it would be of interest to ascertain whether carrots cooked after common methods would yield identical results with raw carrots, in the diet of white rats; *i.e.*, of an animal which is not usually considered as being subject to scurvy, so far as we know at the present time.

*Methods of Experimentation.*

In the first period of feeding, February 6 to March 12, the carrots were scraped or pared, sliced in sections  $\frac{3}{4}$  to 1 inch thick; those fed to Lots I and II were boiled for 30 to 40 minutes in twice their own weight of distilled water. These diets were freshly cooked every other day, and stored in an ice box between the feedings, which occurred twice daily. They were fed without the addition of the juice, which had cooked out, to Lot I; also *with* the addition of this concentrated juice to Lot II. Calorimetric, Kjeldahl, and ash determinations showed an average extraction of about 20 per cent; nitrogen losses were somewhat irregular, being often considerably higher than the losses in salts and in fuel value. During the later period from March 12 up to the present date, when casein, starch, and butter had been added to the carrots, the time of cooking and the amount of water used were increased, and the extraction reached 25 to 30 per cent, so that it equalled that of the average of canned carrots described below.

Carrots were canned by the use of the cold pack method recommended for home canning by the Extension Service of the U. S. Department of Agriculture. This involves 5 minutes blanch of sliced carrots in boiling water, cold dip, packing into glass quart jars, and 2 hours process submerged in bath of boiling water. Our jars contained 700 gm. of carrots (weighed raw) and about 280 cc. of juice remained in the jar at the end of the process. The various tests used showed an extraction of 20 to 40 per cent in different jars and lots, varying with the amount of juice in the jar at the end of processing; average about 28 per cent. Evidently the lessened extraction due to the small amounts of water in the jar almost counterbalanced the tendency of blanching and long processing to increase the degree of extraction above that attained in the ordinary open kettle home cooking process. These canned carrots, after being stored from 3 to 6 months, were fed. to Lot IV, Chart I.

We used commercial casein, soaked 1 week in slightly acidulated water (16 times its own weight, decanted off and renewed once each day), rinsed free of acid; twice extracted with 10 times its own weight of ether, which is decanted off; then spread on a

filter to dry. Leaf lard was rendered and strained through cheese-cloth. Butter fat was heated in a water jacket and passed through filter paper. The starch was Kingsford's cornstarch.

In all cases animals were given as great a quantity of food as they could be induced to consume, and a record was kept of the food consumption of each animal.

### *Cooked Diets Compared to Raw.*

It will be noted that the differences between growth curves of rats fed on raw carrots and those fed on cooked carrots with or without the juice, during the period of exclusive carrot plus salts diet (February 5 to March 12), are perceptible, though not large enough to be highly significant. However, the differences in amount of food consumed in order to make this growth are perhaps significant. The rats fed on cooked carrots without the extracted juice, soon developed a tendency to eat larger amounts of food than did the other two sets, and the same thing was true of the rats fed on canned carrots without the juice. On the other hand, rats fed on cooked carrots plus the juice extracted by cooking consumed somewhat less food than did the other two sets, even at the beginning. These differences in food consumption extended into the period when the carrots fed were fortified by addition of casein plus starch plus butter fat, becoming increasingly greater as time went on; *e.g.*, in 15 to 16 weeks after the beginning of the experiment, rats on raw carrots (fortified), were averaging about 100 gm. of food per day; those on cooked carrots (fortified) plus juice, about 80 gm. per day; those on cooked carrots (fortified) without the juice, 120 gm. per day; those on canned carrots (fortified) without juice, 100 to 110 gm. per day. These rats were in most cases from 150 to 220 gm. in weight, at that time.

During these later periods, percentages of extraction averaged 25 to 30 per cent for both boiled and canned carrots. In the case of the boiled carrots, the juice was more strongly concentrated than before; 3,500 gm. of water were measured out, in which to cook 1,500 gm. of carrots, and after boiling 1 hour the watery juice remaining in the kettle was then concentrated to 200 gm. by slow boiling in an open dish. This was about twice the de-



gree of concentration employed during the first 5 weeks of the feeding period, as far as final bulk of juice was concerned. The rats had shown themselves very fond of this earlier and less concentrated juice, taking it in preference even to the boiled carrots; but they did not like the more concentrated juice, and it had finally to be mixed with the carrots in order to insure their taking it at all. As it had a somewhat rank flavor and also more or less of the taste of caramel or burnt sugar, even when very carefully heated, these flavors were quite perceptible in the diet with which the juice was incorporated.

It is therefore possible to assign several reasons for the failure of the animals on the diet of boiled carrots plus juice, to eat as much food as did the other lots. First, due to some oversight, this lot contained two females and one male, whereas the other two lots contained two males and one female, therefore the three lots are not strictly comparable. It is believed, however, that the lower food consumption of Lot II is not due entirely to this fact. Second, the total bulk of food which must be taken in order to get the same number of calories or grams of protein, is greater in case of boiled carrots plus juice, than in the case of raw carrots, due to the addition of water absorbed by carrots in cooking, and to the water added to the juice which cooked out. It is true that rats are able to increase their food consumption to a remarkable degree as the food becomes more dilute, yet there are limits to their capacity in this direction. Third, their dislike of the strong flavor may have been a cause of gradually diminished appetite. Fourth, it is possible that during the extra period of long heating, especially on the concentrated juice, there was produced some chemical substance which had a depressing effect upon metabolism and therefore upon hunger and appetite. Fifth, it is difficult to explain either the difference in food consumption or that in growth curves as being due to destruction of vitamins by long boiling of the juice in concentrated form; for at the outset, when the quantities of food taken by Lot I, fed on boiled carrots without the addition of extracted juice, and by Lot II, who ate boiled carrots plus the juice, were about equal, the amounts of vitamins taken would have been about equal even if it were to be supposed that the vitamins extracted into the juice were entirely destroyed by heat.

Notwithstanding their larger food consumption, the animals of Lot I (boiled carrots without the juice) had greater difficulty in maintaining themselves in weight equilibrium than did those of Lot III, fed on raw carrots; and the same thing is true of the animals of Lot II (boiled carrots plus juice) and of Lot IV (canned carrots without the juice). This difference is apparent in the first period, when carrots were fed without fortification except by salts, but it is almost altogether lost in later periods, when casein, starch, and butter fat in varying amounts, fortified by a complete salt mixture, were added to the diet. Even when carrot solids formed one-half of the total solids of the diet, the effect of boiling was practically negligible, so far as any influence on the weight curves was concerned. Certainly the cooking of carrots used in human dietaries, when combined with other foods on any scale at present imaginable, cannot be supposed to have deleterious effects, so far as the results of these experiments are concerned. In saying this, we do not forget the interesting experimental work of the Norwegian investigators, Holst and Frölich (3), who report that they were unable to produce scurvy in rats, though animals subject to scurvy showed the deleterious effects of cooking upon antiscorbutic vegetables.

#### *General Effects of Exclusive Carrot Diets.*

All of the exclusive carrot diets produced a constant and considerable degree of diuresis. This diuresis was not altogether due to the large water content of the vegetable since some degree of diuresis was observed in animals fed on a diet of dried carrots. 30 to 45 gm. of urine were collected upon several occasions in 1 day, from a 200 gm. rat. Also the feces were greatly increased in amount and somewhat increased in water content; at times they showed large amounts of undigested matter, particularly in animals not yet accustomed to the diet, and at times there were periods of diarrhea. Rats of 150 gm. or over had much more difficulty when first introduced to the carrot diet, than did those of 40 to 90 gm. All animals during the earlier periods of exclusive carrot feeding showed the familiar symptoms of malnutrition, such as great restlessness, generally increased nervous irritability, emaciation, rough coat, and humped back.

(muscular weakness or perhaps failure of muscles to keep pace with growth of bones). For some days or weeks also, they showed distended bellies, presumably due to distention by undigested carbohydrates or other residue, for there was no unusual accumulation of gas and no abdominal dropsy.

Two litters of rats, born of mothers living on low protein and carrot-rich diets, died at  $2\frac{1}{2}$  and 5 weeks respectively. It seemed that the high degree of diuresis shown by the mothers at that time might have prevented a proper flow of milk.

#### *Nutritive Components of Carrots.*

The carrots used in most of these experiments showed by usual Kjeldahl test about 1 per cent protein ( $N \times 6.25$ ) on the basis of fresh weight (if not at all wilted), or an average of nearly 10 per cent on the basis of dry weight. It is believed to be the case that a large part of this nitrogen does not occur as protein, but that fact need not enter into the discussion here presented.

It is only under the most favorable conditions, and when carrots are fed without dilution of their protein by addition of starch or fat, that the carrot nitrogen is able to maintain body weight in equilibrium without the addition of foreign protein. When circumstances permit, however, not only does maintenance occur upon the exclusive carrots plus salt mixture diet, but there is even an interval between 3 and 8 weeks after the time feeding was started, when there may be a distinct tendency to fatten with increase of weight, although little or no growth of skeleton has occurred (see weight curves of No. 28, Chart II; first 5 weeks of Nos. 1, 10, and 12, Lot III, Chart I).

However, when the proportion of nitrogen in the diet was reduced by adding starch and fat to the carrots (fortified with salts but not with casein or any source of nitrogen), the animals are no longer able to maintain their body weight even for a period.

That carrots contain a not inconsiderable amount of both the water-soluble and the fat-soluble vitamins is evident from the growth curves of Lots I, II, III, and IV, Chart I, and Lots VII and VIII, Chart II. None of these diets contains any source of the water-soluble vitamins, outside of the carrots.<sup>1</sup> All of these

<sup>1</sup> We know this to be the case, for a set of control animals fed with these same lots of purified casein, starch, salts, and butter fat, made up into a

growth curves continue normal through the first generation. Reproduction took place in case of all females, as soon as they were put with the males. The young, however, are not making perfectly normal growth, and the cause of this is still under investigation. Variations in the nutritive values of carrots from different lots are also being studied.

*Dropsy in Albino Rats as Induced by Carrot Diets.*

The problem of diet as related to dropsy is one of greater importance and interest than ever before, since the appearance of "war dropsy" among the victims of malnutrition during the present war. It has been pretty generally ascribed to a lack of fat; but from our experience with albino rats, we are inclined to attach at least equal if not greater importance to the protein or nitrogen content of the diet.

In the mixtures used for feeding rats in Lots XV, XXXI, and XVI, Chart I, the carrot proteins ( $N \times 6.25$ ) constituted between 4 and 5 per cent of the entire dry weight; instead of constituting about 10 per cent, as in the exclusive carrot diets.

Lot XVI was fed on the following diet. Raw chopped carrots, 375 gm.; cornstarch, 30 gm.; complete salt mixture, 3 gm.; lard, 5 gm. No. 76 died at the end of 3 weeks; no dropsy was observed. No. 68 died at the end of 5 weeks with a large amount of fluid in both peritoneal and thoracic cavities. No. 59 died at the end of  $7\frac{1}{2}$  weeks. A large amount of fluid was collected subcutaneously on the chest several days preceding death; this fluid soon escaped freely, wetting the hair along the midventral

synthetic diet, exhibited in their growth curves satisfactory evidence of the absence of the water-soluble vitamine. We also tested another set of control animals with the starch, salts, lard, and purified casein, plus an alcoholic extract of wheat germ; although not all of the symptoms were obtained which we expected in absence of the fat-soluble vitamine (*e.g.*, edema of the eyes), yet there was a striking difference between the growth curves and general appearance of these rats, and those fed on a diet identical with it, except for the replacement of 5 per cent lard by 5 per cent butter fat. Except for a very small amount of fat-soluble vitamine which may be present in some of these purified foodstuffs used as fortifications, carrots are the only source of vitamine in Lots VII and VIII. Further investigations are being made on this point.

line; the amount of fluid found at autopsy was much above normal.

Lot XV was fed the same diet as above except that butter fat replaced lard. Of this lot No. 79 showed the same appearance at autopsy as No. 68 above. Nos. 55 and 75 died at the end of 8 weeks as a result of accident, but had shown no dropsy up to that time. Lot XXXI was fed the same diet as Lot XV above. No. 123 died at the end of 3 weeks, No. 130 at the end of  $7\frac{1}{2}$  weeks, but no dropsy was observed in either case. No. 115 exhibited a remarkable case of dropsy; indeed the weight curve alone shows it most markedly, for there was an increase from 42 to 67 gm. in body weight within a few days before death. This increase was entirely due to the dropsy, as the animal had been steadily losing weight for some time. The day before death the right eye was seen to be badly swollen and almost closed. This swelling gradually spread over the entire body. At autopsy extensive collection of lymph was found under the skin of the legs and of the entire body, also in the thoracic and abdominal cavities.<sup>2</sup>

As yet we have had no true cases of dropsy in rats fed on carrots plus starch plus salts, without the addition of fat although such diets have been running for 10 weeks. In this lot Rats 85 and 86 died at the end of the 8th week. At no time was any marked swelling noticed in either, but in both there was a noticeable swelling of the eyelids a little more than a week before the time of death. From the time the swelling was first noticed, there was an escape of fluid which kept the hair around the mouth and midventral line of the body wet. At autopsy neither of these rats had an abnormal amount of fluid either subcutaneously or in the thoracic or peritoneal cavities, but judging from the rate at which the hair became wet, after being washed and dried, probably 1 or 2 cc. of fluid escaped in this way daily.

There have been no cases of dropsy on fresh, cooked, or dried carrots plus salts. This work on dropsy is very incomplete, but is still in progress and will be reported later.

<sup>2</sup> Autopsies and further study were given all these cases by Dr. H. Gideon Wells, of the Department of Pathology, who will doubtless report his findings in due time.

## SUMMARY.

1. Ordinary methods of cooking do not perceptibly injure the nutritive value of carrots, or certainly not when used as part of a mixed diet. However, a considerable portion of the caloric value of the food is lost when the water used in cooking is rejected, as shown by the greater food consumption of animals on this diet, when the juice in which the carrots were boiled is not added to their food.

2. Carrots when properly supplemented with starch, purified commercial casein, butter or lard, and salts, to such an extent that 50 per cent of the caloric value of the diet is still derived from carrots, will produce normal growth and reproduction in albino rats.

3. Carrots as an exclusive diet, except for the addition of calcium, phosphorus, sodium, and chlorine, may support animals in apparently good health for as long as 16 weeks. Although there is no growth, the animals on this diet may maintain and at times even increase their own body weight. Due to a great variation in the nutritive value of carrots of different lots and in the vitality of different animals, results with this diet are somewhat inconstant.

4. Carrots have a considerable amount of both the water-soluble and fat-soluble vitaminines.

5. Dropsy occurs in a large per cent of rats fed on a carrot diet, when the proportion of nitrogen has been reduced by the addition of some non-nitrogenous foodstuff, such as fat or starch.

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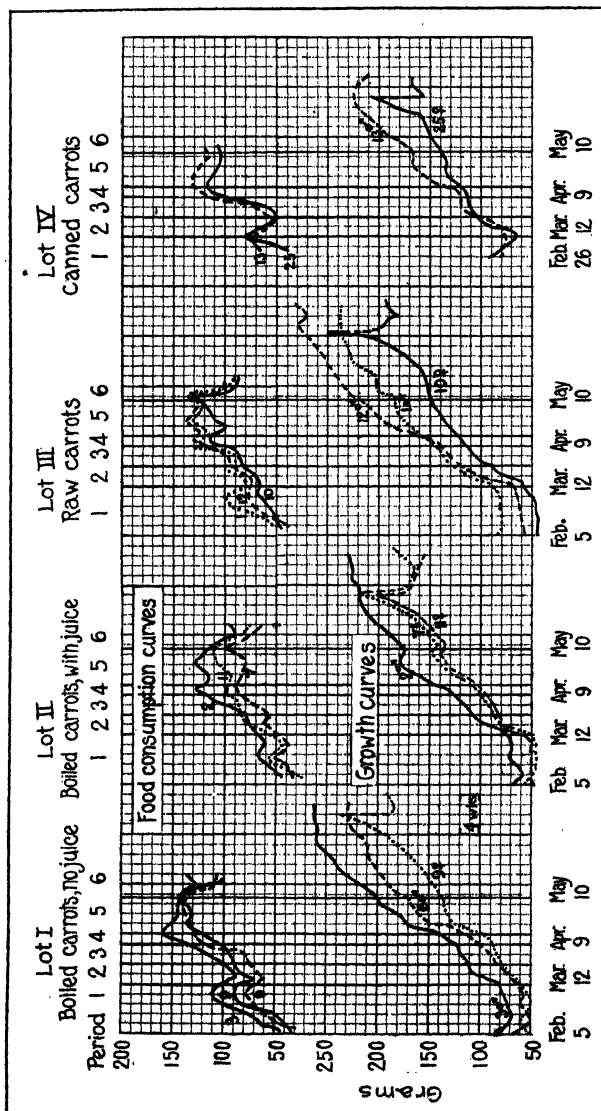


CHART I.

CHART I. Growth curves and food consumption curves. Rations for rats.

Period .....	1	2	3	4	5	6
Carrots, gm....	1,000	1,000	1,000	1,000	1,000	750
Salt II, " ...	4.25	4.25	4.25	4.25	4.25	3.19
Salt III, " ...		3.7	3.7	3.7	3.7	3.8
Starch, " ...		48	24	12	12	37
Casein, " ...		24	12	24	24	24
Butter fat, gm.		20	10	10	10	10
Water for paste, cc.....		400	300	300	150	150

*Salt Mixture II.*

	gm.
Ca lactate.....	1.57
NaCl.....	0.5
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> + 11H <sub>2</sub> O.....	0.6

*Salt Mixture III.*

	gm.
NaCl.....	0.173
Na <sub>2</sub> SO <sub>4</sub> .....	0.318
NaH <sub>2</sub> PO <sub>4</sub> + H <sub>2</sub> O.....	0.347
K <sub>2</sub> HPO <sub>4</sub> .....	0.954
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> + H <sub>2</sub> O.....	0.540
Ca lactate.....	1.300
Fe lactate.....	0.118



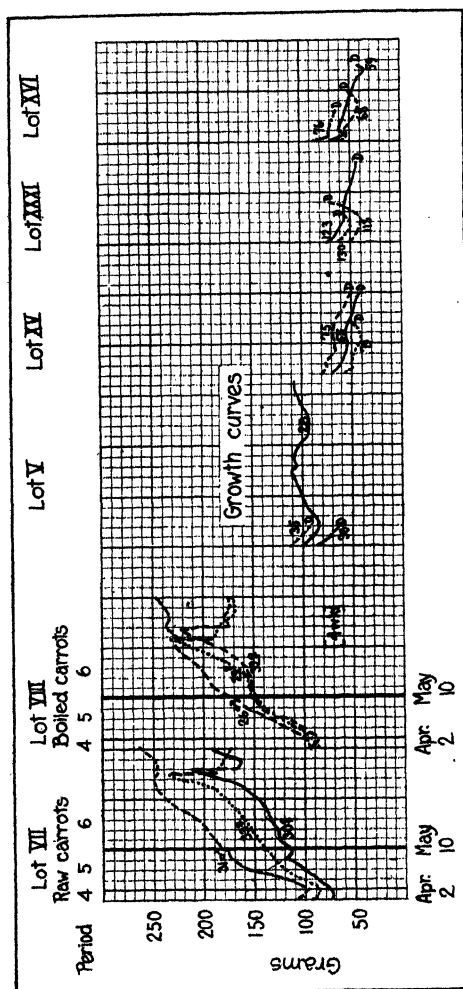


CHART II.

CHART II. Showing growth curves of Lots VII, VIII, V, XV, XXXI, and XVI. Ration for Lots VII and VIII same as for Lots II and III, Chart I, at corresponding periods except that lard was used instead of butter. Ration for Lots V, XV, XXXI, and XVI as follows:

	Lot V.	Lots XV and XXXI.	Lot XVI.
Carrots, <i>gm.</i> .....	100 (Dry.)	375 (Fresh.)	375 (Fresh.)
Salt II, “.....	4.25	1.6	1.6
Starch, “.....		30	30
Salt III, “.....		1.4	1.4
Fat, “.....		5 (Butter.)	5 (Lard.)
Water, <i>cc.</i> .....		75	75



## CREATINURIA. I.

### EXOGENOUS ORIGIN OF URINARY CREATINE.\*

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(Received for publication, September 20, 1918.)

In recent years there is probably no problem of intermediary metabolism in animals that has occupied the attention of, and been subjected to experimental investigations by physiological chemists as much as that of creatine and creatinine. In part this is due to the excellent work of Folin who has made a quantitative determination of these compounds possible, but to a considerable extent it is also due to the apparently many interesting and puzzling relations of these compounds in their origin and fate in the animal body in health and in disease.

It is not intended to present here a lengthy résumé of the general progress that has been made in this field except in so far as in justice to others it becomes imperative to point out in what respects our points of view agree with, or differ from those held by others. In this paper we shall present some of the factors which we believe to be responsible for the apparent anomalies presented by the appearance and disappearance of creatine in the urine under different dietary conditions.

#### *Fasting.*

Creatine was first observed to be a constituent of the urine of fasting man by Benedict<sup>1</sup> and Cathcart.<sup>2</sup> Dorner<sup>3</sup> made the

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

<sup>1</sup> Benedict, F. G., *Carnegie Institution of Washington, Publication* 77, 1907.

<sup>2</sup> Cathcart, E. P., *J. Physiol.*, 1906-07, xxxv, 500.

<sup>3</sup> Dorner, G., *Z. physiol. Chem.*, 1907, lli, 225.

same observation on fasting rabbits. Since then Richards and Wallace,<sup>4</sup> Underhill and Kleiner,<sup>5</sup> Howe and Hawk,<sup>6</sup> and others have obtained corresponding results with dogs, but McCollum and Steenbock,<sup>7</sup> in 1912, found that the pig fasting for as long a period as 14 to 16 days did not excrete creatine. This surprising observation led to the formulation of the thesis that the pig, in comparison with most other animals having greater capacity to store fat, might be possessed of greater ability to use fat during emergencies and thus conserve such other body tissues as would liberate creatine in their disintegration. In an attempt to confirm these observations and establish this hypothesis with additional data we have been surprised to find that the failure of the pig to excrete creatine during fasting, at least with the animals from the University herd, is the exception rather than the rule. To establish the reason for these differences our investigations on creatine excretion have become rather more extensive than originally planned.

Mendel and Rose<sup>8</sup> in studying creatinuria in the rabbit also observed a difference in the behavior of animals with respect to the ease with which creatinuria was induced. One rabbit in an exceptionally good nutritive condition had creatine in its urine in more than traces only as the period of fasting was exceptionally protracted. It is evident, that with careful control of the diet or even withholding of all foods there obtain conditions in the apparently normal animal which determine the presence or absence of creatine in the urine.

Cathcart<sup>9</sup> reported that creatine excretion induced in men by fasting could be reduced to nil by the administration of a diet practically nitrogen- and fat-free. Mendel and Rose<sup>8</sup> criticized Cathcart's results as the diet employed by him was far from having an insignificant nitrogen content, but with the diet absolutely free from nitrogen and fats they obtained with rabbits results the same as Cathcart's.

<sup>4</sup> Richards, A. N., and Wallace, G. B., *J. Biol. Chem.*, 1908, iv, 179.

<sup>5</sup> Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 167.

<sup>6</sup> Howe, P. E., and Hawk, P. B., *J. Am. Chem. Soc.*, 1911, xxxiii, 215.

<sup>7</sup> McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912-13, xiii, 209.

<sup>8</sup> Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911-12, x, 213.

<sup>9</sup> Cathcart, E. P., *J. Physiol.*, 1909-10, xxxix, 311.

That carbohydrate feeding in many instances is able to reduce the amount or even prevent the appearance of creatine induced by fasting is brought out in Table I in our experiments with a pig. A pig was confined in a metabolism cage and 24 hour collections of urine were made. Nitrogen was determined by the Kjeldahl method, and creatine and creatinine by Folin's original method using Benedict's method of dehydration for the creatine. The picric acid used was found to be sufficiently pure to preclude the possibility of introducing an error greater than is ordinarily unavoidable in the method. The analyses were made daily, chloroform and toluene being used as the preservatives.

TABLE I.

Pig, male, weight 23 kilos. Fasted 3 days before collections were made.

Day of collection.	Urinary N.	Preformed creatinine.	Total creatinine.	Creatine as creatinine.	Diet.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
1	5.16	0.425	0.708	0.283	Fasting.
2	6.00	0.302	0.812	0.509	"
3	5.24	0.447	0.836	0.388	"
4	3.66*	0.330*	0.564*	0.234*	"
5	3.51	0.372	0.766	0.422	Starch 500 gm.
6	3.16	0.506	0.581	0.075	" 500 "
7	1.91	0.490	0.490	0.000	" 500 "
8	1.87	0.596	0.679	0.083	" 500 "

\* Incomplete collection.

As is seen in Table I, the ingestion of 500 gm. of starch daily soon resulted in a marked reduction in creatine elimination. Concomitantly there is observed a reduction in nitrogen excretion. The feeding of carbohydrate may have a very pronounced effect on creatine excretion in fasting as indicated above, but this is not necessarily always the case for reasons indicated later. With the pig here under observation the nitrogen of the urine at the beginning of fasting was exceptionally high. This may have accentuated the observed favorable influence of carbohydrate feeding.

*Acidosis.*

Underhill and coworkers,<sup>10</sup> in 1916, on the premises stated by them, "that in nearly every instance in which creatine appears in the urine there is an accompanying acidosis suggests the hypothesis that a condition of acidosis in the body is responsible for the appearance of creatine in the urine," carried out a series of experiments with the rabbit. They concluded "that there is an interrelationship between acidosis and creatine elimination. Creatine in the urine may prove to be an index of a condition of acidosis in the organism." In a later paper they bring out the fact that acidosis is not the only factor responsible for creatinuria as in the phlorhizinized dog administration of sodium bicarbonate leading to the voiding of alkaline urines was without appreciable influence upon the elimination of creatine. Again they state "creatinine may appear in the urine in states of acidosis when carbohydrate deficiency is not involved and creatinuria may be present during carbohydrate deficiency even in the absence of acidosis."

That creatine elimination when occurring in the pig may be dependent at least in part upon an acidosis is brought out in Table II. A fasting pig was given by sound 30 gm. of sodium acetate, dissolved in water, in two portions on 2 successive days. The urine turned alkaline to litmus and the creatine as well as the total nitrogen was immediately reduced. Such data were obtained in many experiments, but as acidosis is not the sole determinant of creatinuria any more than carbohydrate deficiency they will not be presented here *in toto*.

Although creatinuria induced by fasting in the pig can be reduced in degree or even prevented by carbohydrate feeding as well as by the ingestion of virtually or potentially alkaline salts, yet it need not necessarily be prevented by a combination of the two. This is brought out in Table III.

As is observed in Table III the creatine in the urine, while reduced in amount by the ingestion of both carbohydrates and alkalies, is not entirely removed. As the pig was getting a great excess of carbohydrate over that necessary to cover his energy

<sup>10</sup> Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127, 141. Underhill, F. P., and Baumann, E. J., *ibid.*, 147, 151. Underhill, F. P., and Bogert, L. J., *ibid.*, 161.

requirements, and a sufficient amount of sodium acetate to make his urine strongly alkaline to phenolphthalein, certainly then, neither acidosis nor carbohydrate deficiency could be said to be the determinants of creatinuria. With a lower plane of creatine

TABLE II.

Pig, male, weight 25 kilos. Fasted 3 days before collections were made.

Day of collection.	Urinary N.	Preformed creatinine.	Total creatinine.	Creatine as creatinine.	Diet.
	gm.	gm.	gm.	gm.	
1	11.38	0.312	1.311	0.999	Fasting.
2	9.72	0.318	0.991	0.673	"
3	4.16	0.324	0.469	0.145	Sodium acetate 30 gm.
4	4.13	0.383	0.501	0.118	" " 30 "

TABLE III.

Pig, male, weight 27 kilos. Fasted 2 days before collections were made.

Day of collection.	Volume of urine.	Urinary N.	Reaction of urine.	Ammonia N.	Preformed creatinine.	Total creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.		gm.	gm.	gm.	gm.	
1	675	11.41	Acid.*	0.935	0.574	0.957	0.382	Fasting.
2	450	8.25	" "	0.603	0.438	0.877	0.438	"
3	360	8.08	" "	0.817	0.434	0.852	0.417	"
4	600	7.06	Alkaline.†	0.115	0.648	0.879	0.231	Starch 250 gm. Na acetate 30 gm. Starch 500 gm. Na acetate 20 gm. Starch 500 gm. Na acetate 20 gm. Starch 500 gm. Na acetate 20 gm.
5	1,625	3.94	" †	0.248	0.603	0.774	0.171	
6	2,200	3.22	" †	0.267	0.509	0.774	0.264	
7	1,750	3.54	" †	0.148	0.653	0.854	0.200	

\* To litmus.

† To phenolphthalein.

elimination from that observed in this experimental animal we have every reason to believe that instances may occur where reduction of the acidosis or removal of carbohydrate fasting singly or jointly may lead to the total disappearance of the creat-



inuria. It should be emphasized that here again the reduction of the creatinuria was accompanied by a marked decrease in the nitrogen excretion.

In an experiment carried out previous to those already cited we had made the attempt in a fasting pig, which failed to show

TABLE IV.

Pig, male, weight 35 kilos. Collections started on the 5th day of fasting.

Day of collection.	Volume of urine.	Urinary N.	Acidity of 25 cc. urine. Cc. N/14 NaOH.	Ammonia N.	Creatinine.	Creatinine as creatinine.	Diet.
	cc.	gm.		gm.	gm.	gm.	
1	1,260	3.57	5.7	0.257	0.833	0.000	Water.
2	1,440	2.88	4.1	0.201	0.761	0.000	" "
3	670	2.76	10.1	0.201	0.798	0.000	"
4	700	2.67	6.7	0.145	0.833	0.000	"
5	1,405	3.06	3.0	0.320	0.759	0.000	" +2.5 cc. c.p. HCl.
6	1,350	2.54	3.5	0.459	0.624	0.000	"
7	1,490	3.22	4.3	0.643	0.946	0.000	" + 10 cc. c.p. HCl.
8	1,440	2.37	3.4	0.869	0.864	0.000	"
9	475	1.41	6.3	0.249	0.499	0.000	" + 10 cc. c.p. HCl.*
10	1,530	3.39	2.9	0.881	0.991	0.000	"
11	1,385	1.95	2.6	0.426	0.669	0.000	"
12	1,470	1.75	2.2	0.352	0.744	0.000	"
13	1,537	1.94	2.0	0.325	0.748	0.000	"
14	1,460	2.47	2.7	0.671	0.705	0.000	" + 10 cc. c.p. HCl.
15	1,585	2.85	3.5	1.109	0.703	0.000	" + 10 " " " HCl.
16	1,450	2.51	2.4	0.986	0.700	0.000	" + 5 " " " HCl.*
17	1,535	2.41	2.5	0.749	0.656	0.000	"
18	1,545	2.16	2.0	0.568	0.625	0.000	"
19	1,515	2.06	2.1	0.412	0.613	0.000	"

\* Part of acid lost by vomiting soon after administration. On the 16th day of collection at least one-half of the acid was lost in this way, but collections of urine were not contaminated.

creatinuria, to produce creatinuria by the administration of an inorganic acid. That our attempts were entirely unsuccessful, even though 5 and 10 cc. of concentrated hydrochloric acid were given on successive days, is shown in Table IV.

The failure to induce creatinuria in this animal led us to attempt to repeat the experiment. We were, however, unable to

find another pig which did not have creatine in its urine, so that our endeavors were necessarily confined to attempts to increase the creatine excretion of animals already excreting creatine in the urine. In the experiment presented in Table IV we were impressed with the difficulty of producing a strongly acid-reacting urine even when as much as 10 cc. of concentrated hydrochloric acid were given to the fasting animal. It is true that our only measure of the acidity of the urine was a titrimetric measure of its alkali-absorbing capacity rather than its hydrogen ion concentration, but in a rough way under many biological conditions such values have been shown to run sufficiently parallel for comparative results.<sup>11</sup> For determination of the degree of acidity we have in later experiments determined the hydrogen ion concentration of the urine by the colorimetric method using Clark's standards.<sup>12</sup> In all cases the acidosis induced by acid administration was but small as compared with the values on rabbits obtained by Underhill.<sup>10</sup> We secured additional evidence of the tendency to an acidosis, however, by the tremendous increase in the values for ammonia. In some cases we also followed the phosphorus excretions.

As seen in Table V, upon the administration of 5 cc. of concentrated hydrochloric acid on 2 days the hydrogen ion concentration was increased slightly, the ammonia increased greatly, yet the creatine did not increase; in fact, on 1 day after the acid was given the creatine disappeared entirely. With this animal the amount of acid given could not be increased as such attempts led to immediate regurgitation of stomach contents.

In Table VI a pig was given 1.53 gm. of hydrochloric acid diluted to 1,200 cc. with distilled water. The acid was given with a sound in four portions during the course of the day. The acid was traced to the urine by the observed increased chloride excretion as determined by the Volhard-Arnold method. When acid was given there was a general increase in the hydrogen ion concentration of the urine although the effect was not observed immediately from day to day. On 3 out of the 4 days when acid was given there was a decided increase in creatine elimina-

<sup>11</sup> Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1914, xvii, 305.

<sup>12</sup> Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917 ii, 109, 191.

TABLE V.

Pig, male, weight 36 kilos. Collections started on the 8th day of feeding the starch diet.

Day of collection.	Volume of urine. *	Urinary N.	Ammonia N.	pH	Creatinine.	Creatine.	Diet.
	cc.	gm.	gm.		gm.	gm.	
1	3,200	1.86	0.193	6.4	0.649	0.191	Starch 300 gm.
2	2,150	1.59	0.189	6.4	0.731	0.137	" 300 "
3	1,750	1.53	0.257	6.4	0.901	0.130	" 300 "
4	1,850	1.85	0.740	6.0	0.889	0.147	" 500 " + 5 cc. C.P. HCl.
5	1,700	1.70	0.605	6.4	0.892	0.000	" 500 "
6	1,900	2.01	0.866	6.0	0.836	0.114	" 500 " + 5 cc. C.P. HCl.
7	1,375	1.86	0.825	6.2	0.896	0.101	" 500 "

TABLE VI.

Pig, male, weight 18 kilos. Fed on 350 gm. of starch daily 5 days before collections were made, but on the first 2 days food was refused, on the next 2 days it was partly consumed, and only on the last day just previous to the beginning of the record it was all consumed.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	pH	Creatinine.	Creatine as creatinine.	Chlorides as HCl.	P <sub>2</sub> O <sub>5</sub>	Diet.
	cc.	gm.	gm.		gm.	gm.	gm.	gm.	
1	510	1.63	0.414	7.4	0.327	0.032	0.01	0.54	Starch.
2	550	1.56	0.295	7.2	0.396	0.035	0.09	0.33	"
3	1,910	1.86	0.609	6.3	0.399	0.130	1.04	0.32	" + HCl 1.53 gm.
4	500	1.37	0.378	6.5	0.398	0.035	0.62	0.30	"
5	1,675	2.45	0.610	6.5	0.399	0.121	1.29	0.61	" + HCl 1.47 gm.
6	450	1.90	0.521	6.3	0.455	0.065	0.53	0.53	"
7	310	1.47	0.329	6.1	0.448	0.045	0.13	0.53	"
8	1,600	1.83	0.576	6.5	0.483	0.078	0.78	0.54	" + HCl 1.53 gm.
9	1,600	1.88	0.650	5.9	0.385	0.115	1.40	0.61	" + HCl 1.53 "
10	370	1.49	0.532	5.8	0.457	0.035	0.56	0.38	"
11	310	1.88	0.444	6.0*	0.405	0.100	0.35	0.46	"

\* On the 2 following days as the starch diet was continued the pH had come back to 6.5 and 6.4 respectively.

tion. This indicates that acid administration, even in the pig with its tremendous capacity for counteracting the tendency to establish acidosis, can cause an increase in creatine excretion. During such times when the creatine did not increase it may be surmised that the acid-neutralizing mechanism may have been so efficient that the threshold for increased creatine production was not crossed. Weight is given to this point of view by the fact that on such days the hydrogen ion concentration of the urine was not very high. Our data are not of the character to establish this definitely, but nevertheless we believe them to be strongly suggestive of this point of view. Whether by virtue of the comparatively slight increase in hydrogen ion concentration or the great increase in ammonia excretion, we are willing to grant that an acidosis obtained; the fact nevertheless is clear that acid administration to the extent that it is tolerated by the animal may or may not increase creatine elimination. Acidosis and carbohydrate deficiency singly or jointly play a rôle in certain forms of creatinuria.

#### *Protein Feeding.*

In 1911, when Mendel and Rose<sup>8</sup> confirmed Cathcart's<sup>9</sup> observations that creatinuria may be prevented by carbohydrate feeding, they also stated that, "The metabolism of exogenous or reserve proteins is not accompanied by the production of creatine or creatinine." Just why they should reach this conclusion is not quite clear as their experimental data were not of the character to bring out this point conclusively. In 1912, Folin and Denis<sup>13</sup> in verifying Rose's findings that creatine is usually present in the urine of children, even on a creatine-free diet, stated: "We are inclined to believe that the creatine in children's urine does not depend . . . on a peculiar carbohydrate metabolism but that it is due to an excessively high level of protein consumption (in proportion to mass of muscles in the body)." Furthermore, they said, "If the above hypothesis is correct it should be possible to reproduce in adults by forced feeding with protein which contains no creatine the conditions with reference

<sup>13</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 253.

to creatine found in children and it should also be possible to obtain creatine free urine from children by reducing their protein consumption." Shortly afterwards it was shown in this laboratory<sup>7</sup> that with the pig ingestion of protein from different sources could lead to decided differences in creatine excretion. The kind as well as the amount of protein ingested seemed to be important in this connection. Powis and Raper<sup>14</sup> concluded: "Assimilation of food has little or no influence on creatine excretion." An increased creatine excretion during the day as compared with that during the night was attributed by them to differences in state of rest of the skeletal muscles. Denis<sup>15</sup> was, however, unable to confirm their results. She found the creatine excretion highest after heavy meals and therefore still adhered to the theory of exogenous origin of creatine. Later Denis and Kramer<sup>16</sup> proved one of the points which Folin and Denis<sup>13</sup> had postulated as being necessary to establish the exogenous origin of creatine. They expressed themselves as follows: "We have found that by feeding children diets extremely low in protein but of sufficient calorific value, it is possible to obtain urines absolutely creatine-free. On feeding increasing amounts of protein, creatine excretion begins, increases day by day, and if the protein intake can be pushed to a sufficiently high point may become considerably greater than the excretion of preformed creatinine." Denis and Minot<sup>17</sup> subsequently also satisfied the other requisite demanded by the theory; that is, they were able to produce creatinuria in adults by feeding a high protein diet. It should, however, be mentioned that normal men in contrast with women presented a peculiar problem in that creatinuria has not yet been induced in them by high protein feeding. It has, however, been induced in a man afflicted with hyperthyroid activity. We shall discuss this later.

In spite of the evidence cited above, Rose, Dimmitt, and Bartlett,<sup>18</sup> while this paper was in preparation, have stated

<sup>14</sup> Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 362.

<sup>15</sup> Denis, W., *J. Biol. Chem.*, 1917, xxix, 447.

<sup>16</sup> Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.

<sup>17</sup> Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1917, xxxi, 561.

<sup>18</sup> Rose, W. C., Dimmitt, J. S., and Bartlett, H. L., *J. Biol. Chem.*, 1918, xxxiv, 601.

"that the theory which attributes the excretion of creatine on a creatine-free diet to an exogenous origin in the protein of the diet is untenable." Apparently they arrived at this sweeping conclusion on the basis of their experiments in which they were unable by high protein feeding to produce creatinuria in men or to increase it in women.

In the experiments which follow we shall indicate again that creatine in the urine can be caused to appear or if present can be caused to increase in amount as the result of no other modification in the diet than the introduction of creatine-free protein. In Table VII we have a continuation of a record of performance of a pig fasted for 23 days, as tabulated in Table IV. It was fed 200 gm. of casein on the 1st day and 400 gm. for 4 consecutive days thereafter. It was then fasted, water always being given *ad libitum*. This record indicates with what remarkable persistence the absence of creatine may be maintained in spite of the long preliminary fasting period with acid ingestion. The final appearance of creatine with the ingestion of abnormally large amounts of protein, as well as its subsequent rapid disappearance when protein feeding was discontinued, is perfectly definite, and scarcely leaves room for the argument that the very resistance to inception of creatinuria by protein feeding should speak against high protein as a major factor in creatinuria production. In the case of this animal there is no doubt that smaller amounts of protein might have been fed even for a considerable time without the appearance of any creatine.

The situation was entirely different with the animal recorded in Table VIII. This pig which normally had enormous quantities of creatine in its urine doubled the excretion of its creatine when it was given only 200 gm. of casein. It may be stated that whenever creatine was already being excreted by an animal the amount of protein necessary to produce a certain amount of creatine was far less than when it was originally absent. In speaking of creatine production it should not be forgotten that the amount of creatine produced is undoubtedly not synonymous with the amount excreted.

In the experiments detailed in Tables VII and VIII it was possible that the creatine elimination was indirectly due to the stimulation of endogenous metabolism resulting from the sulfuric

and phosphoric acids produced in the metabolism of the casein. To determine the rôle of these factors we next gave a number of

TABLE VII.

Same pig used as for Table IV, the collections being continuous. Weight of animal 28 kilos.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.	gm.	gm.	
20	1,100	3.20	0.318	0.667	0.000	Casein 200 gm.
21	1,180	12.44	0.900	0.820	0.000	" 400 "
22	1,430	21.90	1.779	0.780	0.095	" 400 "
23	1,910	33.62	2.493	0.799	0.370	" 400 "
24	1,925	35.37	2.701	0.715	0.683	" 400 "
25	1,700	32.55	2.318	0.678	0.249	Fasted.
26	1,130	16.19	1.056	0.695	0.051	"
27	1,700	7.00	0.520	0.791	0.000	"
28	1,415	3.18	0.331	0.736	0.000	"

TABLE VIII.

Pig, male, weight 24 kilos. Previous to the beginning of the collections recorded in this protocol the pig fasted for 8 days, on one of which, 4 days before the collections tabulated below were made, it received 30 gm. of sodium acetate.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to litmus.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.		gm.	gm.	
1	1,250	4.83	0.256	Slightly alkaline.	0.460	0.481	Fasting.
2	1,640	4.41	0.271	" "	0.563	0.429	"
3	1,925	4.05	0.309	" "	0.482	0.539	"
4	2,675	8.14	0.480	" "	0.424	0.924	Casein 200 gm.
5	3,500	14.78	0.464	" "	0.328	1.094	Fasting.
6	1,400	5.46	0.403	" "	0.333	0.344	"
7	2,700	6.96	0.208	" "	0.307	0.614	"
8	2,200	3.94	0.506	Neutral.	0.321	0.524	"

\* Almost neutral.

pigs an amount of sodium acetate sufficient to keep their urine strongly alkaline, especially on the days when the casein was fed. The data of such experiments are recorded in Tables IX, X, and

XI. They were obtained on the same animals as those already presented in Tables I, II, and III respectively.

That the rôle of inorganic acid having its origin in the metabolism of exogenous protein is a minor or even a negligible one in creatine production, we have proved to our satisfaction in still another way. It was found that while protein feeding would cause an increase in creatine excretion, amounts of hydrochloric acid equivalent to the potential acidity of the sulfur of the in-

TABLE IX.

Same pig used as for Table I. After the completion of that record the starch diet was continued the following 5 days before the collections tabulated below were made. On the 2 days immediately previous to this record 20 gm. of sodium acetate were given daily.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to phenolphthalein.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.		gm.	gm.	
1	1,820	1.33	0.047	Alkaline.	0.569	0.142	Starch 500 gm. Na acetate 20 gm. Starch 500 gm. Na acetate 20 gm. Starch 500 gm. Na acetate 22.5 gm. Casein 100 gm. Starch 500 gm. Na acetate 17.5 gm. Starch 500 gm. Na acetate 20 gm.
2	1,450	1.82	0.021	"	0.512	0.239	
3	1,025	3.97	0.120	"	0.520	0.546	
4	1,250	3.21	0.214	Acid.*	0.517	0.169	
5	1,350	3.25	0.071	Alkaline.	0.514	0.319	

\* Alkaline to litmus.

gested protein were entirely ineffective. Needless to say, these experiments in comparative efficacy of protein and its acidity in creatine production had to be carried out on the same animals and furthermore could only be demonstrated with animals already excreting creatine. With others such as those of which the records were presented in Tables IV and VI the amount of protein necessary to produce creatinuria is so large that when attempts were made to give equivalent amounts of acid, vomiting resulted.



TABLE X.

Same pig used as for Table II. Only 1 day intervened between the two records, during which there was no change in diet.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to phenolphthalein.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.		gm.	gm.	
1	1,750	2.81	—	Alkaline.	0.548	0.208	Starch 500 gm. Na acetate 30 gm. Starch 500 gm. Na acetate 30 gm. Starch 500 gm. Na acetate 50 gm. Casein 200 gm. Starch 500 gm. Na acetate 40 gm. Starch 500 gm. Na acetate 30 gm. Starch 500 gm. Na acetate 30 gm.
2	1,550	1.69	0.067	"	0.604	0.212	
3	1,400	5.75	0.154	"	0.623	0.811	
4	1,720	7.29	0.152	"	0.573	0.643	
5	1,500	3.58	0.063	"	0.770	0.137	
6	1,500	2.17	0.064	"	0.787	0.169	

TABLE XI.

Same pig used as for Table III. The first 2 days of this record are taken from Table III to bring out the effect of casein feeding on the creatine increase.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to phenolphthalein.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.		gm.	gm.	
1	2,200	3.23	0.268	Alkaline.	0.509	0.265	Starch 500 gm. Na acetate 20 gm. Starch 500 gm. Na acetate 20 gm. Starch 500 gm. Na acetate 30 gm. Casein 100 gm. Starch 500 gm. Na acetate 30 gm. Starch 500 gm. Na acetate 20 gm.
2	1,750	3.54	0.148	"	0.654	0.200	
3	1,750	8.96	0.169	"	0.642	0.578	
4	1,620	4.25	0.232	"	0.602	0.128	
5	1,800	3.61	0.154	"	0.640	0.164	

In the calculations, sulfur was assumed to be completely oxidized to sulfuric acid. The results of one such experiment are presented in Table XII.

TABLE XII.

Pig, male, weight 13.6 kilos. The pig was fasted for 3 days. It then ate 250 gm. of starch in the form of a paste daily for 6 days, on one of which, 2 days before the collections tabulated below were made, it received 0.75 gm. of HCl in addition. 600 cc. of distilled water with or without the acids were given daily by means of a sound.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Creatinine.	Creatine as creatinine.	Chlorides as HCl.	P <sub>2</sub> O <sub>5</sub> .	Diet.
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	
1	1,000	0.98	0.316	0.286	0.055	0.538	0.279	Starch 250 gm.
2	1,000	0.73	0.203	0.270	0.081	0.359	0.233	" 250 "
3	1,000	0.78	0.298	0.289	0.048	1.172	0.178	" 250 "
4	1,000	0.84	0.497	0.307	0.052	1.192	0.189	HCl 0.76 gm. Starch 250 gm.
5	700	1.28	0.529	0.317	0.052	0.414	0.348	HCl 1.52 gm. Starch 250 gm.
6	650	4.69	0.575	0.308	0.161	0.652	0.322	" 158 "
7	1,025	3.99	0.504	0.345	0.620	0.433	0.378	Edestin 83.3 gm.* Starch 250 gm.
8	950	1.96	0.369	0.313	0.074	0.348	0.258	" 250 "

\* The edestin was not a pure preparation, but contained some carbohydrate which reduced its N to 14.55 per cent and its S to 0.71 per cent. Calculated over into H<sub>2</sub>SO<sub>4</sub> its S would be equivalent to 1.31 gm. of HCl in acidity.

## DISCUSSION.

\*We have now demonstrated to our satisfaction that in the pig, creatine in the urine may or may not be produced by fasting. From data previously presented as well as from later occasional observations we have noticed that animals giving every external appearance of being normal may or may not have creatine in the urine on such rations as it is customary to feed in good animal husbandry practice. We have also shown that the creatinuria obtaining during fasting may be reduced by carbohydrate feeding or by the administration of alkali, but that both treatments applied simultaneously need not necessarily prevent it.

In addition, we are convinced that acid administration resulting in a slight acidosis may or may not increase the creatine, and that protein feeding, if sufficiently intensive, will always produce creatinuria or if already present will increase it in degree. This effect obtains even during alkalosis and cannot be attributed to acids resulting from the metabolism of the protein molecule.

We realize fully that our conclusions are at variance with many that have been made on an apparently unimpeachable experimental basis and we realize also that in the contentions here presented it may appear as though confusion in the study of creatine metabolism has been increased rather than diminished. On critical analysis this shows itself to be far from the case. We are convinced that in one form or another creatinuria is etiologically related to protein metabolism, whether of exogenous or endogenous origin, and that in addition, in a manner as yet unknown, it is related to the creatine stored in the muscles and other tissues as well. With this fundamental conception it becomes possible to correlate all reliable observations hitherto made on creatine excretion.

Since Folin,<sup>19</sup> in 1906, showed that creatine ingested by man did not often make its appearance in the urine or even increase the nitrogen excretion, the idea has been quite prevalent that ingested creatine is absorbed by the muscles and that creatinuria is then related to the degree to which saturation of the muscle obtains. Thus Denis<sup>20</sup> in discussing the effect of high protein diets stated: "It is exceedingly improbable, however, that any creatine excretion would result were the diet given to these hyperthyroid patients eaten by a normal woman. It would therefore seem possible that in this condition and in some other pathological conditions, as well as in childhood, the saturation point of the muscle for creatine is low, a hypothesis supported by the low values for muscle creatine found in children and in persons suffering from exophthalmic goiter." We can hardly see the force of this argument though we readily accept the conception of creatine storage and consider that it probably is a misstatement as Denis previously stated in the same article: "As a working hypothesis it may be suggested, therefore, that on the ingestion

<sup>19</sup> Folin, O., *Festschrift Olof Hammarsten*, Upsala, 1906, paper iii

<sup>20</sup> Denis, *J. Biol. Chem.*, 1917, xxx, 47.

of protein some fraction of this is transformed into creatine, transported to the muscles, and there absorbed. If so much creatine is manufactured that the muscles become supersaturated, creatine is excreted by way of the kidney." Again, Rose and coworkers<sup>18</sup> state: "Accordingly, the creatine excretion in children, and in adults with exophthalmic goiter may be due not only to the high protein of the diet, but perhaps also to a low saturation point of the muscle for creatine."

The creatine content of the muscle in its relation to creatinuria obtaining as it often does over long periods of time must be secondary in importance. While a certain amount of creatine found in the urine can have its origin in the creatine of the tissues, in the final analysis of creatinuria we must consider from what source of materials even that creatine was originally formed. Reduced to its simplest terms the present immediate problem resolves itself into a consideration of the mechanism of the formation of all creatine, as in the light of present experimental data we are not warranted in assuming that the creatine of the muscles owes its origin to different metabolic processes from that of the creatine excreted in the urine.

Creatine is formed from precursors in the protein molecule. Whenever the protein molecule is disintegrated, these precursors are liberated and to the extent that they are not utilized in the body an attempt is made to utilize their energy. In part, this results in the formation of urea but, as with the guanidine residue in arginine, urea formation to the exclusion of the formation of other nitrogenous decomposition products is not possible. Arginine may be catabolized by two essentially different processes. It may be split into urea and ornithine, which ultimately is also deaminated and excreted as urea, or it may be split into components leaving the guanidine grouping intact. In the latter instance cleavage would occur at the linkage of the carbon chain with the imino group, or the carbon chain would be deaminated and then oxidized to a two-carbon residue,—acetic acid. In the one case guanidine would be formed which would be acetylated—as guanidine is exceedingly toxic, while guanidine acetic acid is practically innocuous; in the other case we would already have guanidine acetic acid; in either case methylation would result in the formation of creatine. The biological methylation of guani-

dine acetic acid has been demonstrated experimentally,<sup>3,21</sup> so that this step offers no difficulty for conception, but we are not prepared to say by which of the two methods the formation of guanidine acetic acid occurs. In the *intra vitam* synthesis of creatine Henderson's<sup>22</sup> observations are of interest. She found that in parathyroidectomized dogs a decrease in the free guanidine of the muscle tissue corresponded well with an observed increase in creatine. At any rate, according to our hypothesis creatine is an end-product in the catabolism of certain precursors in the protein molecule just as urea is of others.

The above conception may be challenged on the basis of data pointing to the great variability in the amount of creatine excreted under different conditions and especially on account of the lack of a definite ratio between the total nitrogen and the creatine nitrogen. Howe and Hawk<sup>6</sup> stated: "That there is a direct relation between the amount of nitrogenous material catabolized and the creatine excreted is brought out in our results." But later<sup>23</sup> they point out the discrepancy in the amount of nitrogen eliminated and the creatine nitrogen if referable to complete muscular disintegration. In proportion to creatine nitrogen the total nitrogen should have been higher. Benedict and Osterberg<sup>24</sup> stated: "There is no parallelism between body tissue destroyed, and creatine elimination. Sparing the body nitrogen does not affect the creatine output." And again they<sup>24</sup> may be quoted: "Myers and Fine have recently suggested that the reason carbohydrate is effective in causing cessation of creatine elimination is because of the sparing action on protein metabolism. Such an explanation would be more satisfactory were it not that the creatine wholly disappears upon a pure carbohydrate diet, while only a portion of the muscle tissue can be spared by such a diet." M. S. Rose<sup>25</sup> also found that in women, creatine elimination in the urine is not definitely influenced by protein in the diet.

<sup>21</sup> Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430.

<sup>22</sup> Henderson, P. S., *J. Physiol.*, 1918, lii, 1.

<sup>23</sup> Howe, P. E., Mattill, H. A., and Hawk, P. B., *J. Biol. Chem.*, 1911-12, x, 417.

<sup>24</sup> Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1914, xviii, 195.

<sup>25</sup> Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1.

We believe that the above arguments can be successfully answered from information available as a result of modern investigations on protein metabolism, keeping in mind the possibility that the metabolism of creatine precursors may proceed in different directions and that the creatine precursors during maintenance, but especially during growth, during body tissue restitution, and during milk production are utilized and not completely metabolized to creatine.

It is significant that creatine generally occurs in the urine in instances where there is an increased protein catabolism; *viz.*, during fevers, in exophthalmic goiter, in tumor cachexia, in post partum resolution of the uterus, in hydrazine and phosphorus poisoning, and in diabetes mellitus. It is also significant that creatine in the urine is reduced or prevented by just those agents which reduce protein catabolism. For instance, carbohydrates and alkaline salts both reduce protein catabolism and both reduce creatinuria; fats do not reduce protein catabolism and correspondingly do not reduce creatinuria. With respect to carbohydrates this conception is not new, as Shaffer and Coleman<sup>26</sup> in 1909 found that diets rich in carbohydrates in the treatment of typhoid prevented the loss of creatine or reduced it to a minimum which they attributed to a body protein-sparing action. Myers and Fine<sup>27</sup> in 1913 stated: "The decreased elimination of creatine after feeding carbohydrate is primarily dependent upon the sparing action of carbohydrate . . . on protein metabolism." But further than this they did not go.

With protein feeding the matter is apparently far different, but actually it is analogous. It becomes a question of supplementation of amino-acids in such a manner that after the demands of the body are satisfied no creatine precursors are left over to be metabolized into creatine.

Let us take as our first example for the discussion of the effect of protein feeding on creatinuria, the case of the actively growing animal. For growth, an animal has need in the protein of its food of a certain amount of those amino-acids which it cannot synthesize. An example of such an amino-acid we have in

<sup>26</sup> Shaffer, P. A., and Coleman, W., *Arch. Int. Med.*, 1909, iv, 538.

<sup>27</sup> Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 305.

lysine. If an ingested protein is primarily poor in lysine and unable to cover the requirements of the growing animal for it, then the other amino-acids, even if they are present in proper amounts and proportions to make the new protein molecule, cannot all be retained. They are retained only to the extent that the growth determinant lysine makes this possible. The excess is metabolized to obtain its energy and the nitrogen is excreted as waste in the urine. Some of this nitrogen appears as urea and some of it, if creatine precursors are present in excess relative to the demands for protein synthesis, as creatine. The inevitable result of such supplementary relations would be, that a fortunate selection of kind as well as amount of protein would lead to creatine production in one case and to a suspension of its formation in another. This would depend on whether in the supplementary relations all or only part of the creatine precursors in the system could be used up in the synthesis of the new protein molecule. Any factor influencing growth would affect the creatine excretion.

In periods of excessive endogenous protein metabolism leading to the elimination of creatine, the action of feeding protein with respect to its effect on the creatinuria is entirely similar. Mendel and Rose<sup>8</sup> with fasting rabbits found that creatine elimination was not prevented by feeding fat and protein, but Osterberg and Wolf,<sup>28</sup> Benedict and Osterberg,<sup>24</sup> and later Rose<sup>29</sup> found that in the dog creatinuria induced by fasting was inhibited by protein feeding. Rose and coworkers<sup>30</sup> also made the same observation during fasting in man, but Cathcart,<sup>9</sup> who first studied this problem, did not find it so when giving protein and fat. The Cornell investigators, as well as Rose, were inclined to attribute the disappearance of creatine to the action of the glucose which could have its origin in the protein molecule. The administration of an equivalent amount of glucose was observed to have the same effect and moreover in diabetes with failure of ability to utilize the carbohydrate which might have its origin in the protein molecule, protein feeding was ineffective. It may well

<sup>28</sup> Osterberg, E., and Wolf, C. G. L., *J. Biol. Chem.*, 1908, iv, p. xxiii.

<sup>29</sup> Rose, W. C., *J. Biol. Chem.*, 1916, xxvi, 331.

<sup>30</sup> Rose, W. C., Dimmitt, F. W., and Cheatham, P. N. *J. Biol. Chem.*, 1916, xxvi, 339.

be that part of the action of the protein can be attributed to this factor, but we believe that one more important is to be found in the supplementary relations of the amino-acids ingested to those liberated in endogenous protein catabolism. It is now demonstrated that for repair processes during maintenance the amino-acid demands are different from those during growth. This must mean as has been expressed before from this laboratory<sup>31</sup> that in endogenous protein catabolism the destruction of all amino-acids is not in proportion to the extent in which the individual amino-acids occur in the disintegrating protein molecule. As the body protein is catabolized creatine precursors are liberated and creatine appears in the urine. On feeding protein the ingestion of sufficient amounts of those amino-acids already destroyed makes possible the retention of the creatine precursors in protein synthesis and creatine disappears from the urine. If, however, the ingested protein is unusually rich in creatine precursors then creatine does not disappear from the urine as excessive amounts of it will now be formed from the exogenous protein metabolism.

In the lactating animal we have again the same though slightly modified conditions. Mellanby<sup>32</sup> observed that: "The increase in weight of healthy children breast fed under similar normal conditions is roughly proportional to the amount of creatine in the mother's urine. In other words, the creatine excreted in the urine seems to have some relation to the nutriment given by the mother to her child." Apparently we have here another instance where the relation of creatine production to protein catabolism is evident. We may accept that the creatine here observed has its origin in the unusually large amounts of protein consumed by the lactating mother, of which the excessive amounts of creatine precursors are metabolized to creatine, but creatinuria in this case might also owe its origin to the disproportionate amounts of creatine precursors ingested to the sum total of the requirements imposed by body protein conservation and milk protein production.

In the foregoing, while we have often spoken of both creatine production and creatine excretion we have held in abeyance a

<sup>31</sup> McCollum, E. V., *Wisconsin Exp. Station, Research Bull.* 21, 1912.

<sup>32</sup> Mellanby, E., *Proc. Roy. Soc., Series B*, 1913, lxxxvi, 88.



discussion of the discrepancies which may obtain between the amounts of creatine produced and the amounts of creatine excreted. Until, as Folin and Denis<sup>33</sup> have emphasized, more data are available on the variation in the creatine content of body tissues, it is not possible to discuss this phase of the problem at length, but we do believe that many of the interpretations of data on creatine production and elimination are faulty because of failure to take cognizance of the fact that creatine may in part be utilized or destroyed.

That the animal body has a fairly definite ability to destroy creatine and that in creatinuria creatine has been produced in such amounts that the ability of the body to destroy it has been exceeded, is especially suggested by Folin's<sup>19</sup> observation that creatinuria could be induced far more readily by creatine feeding in man when the subject was receiving a high protein diet than when receiving a low protein diet. Under such circumstances while the creatine ingested did not exceed in amount the ability of the subject to destroy it, the sum total of the creatine ingested together with that having its origin in the protein did exceed it and creatinuria resulted.

Apparently, however, Folin or Denis<sup>33</sup> are not inclined to consider creatine a waste product, as they have stated: "Creatine must, however, be a waste product or a synthetic product serving some special function or as a synthetic product it may in fact be a part of the active living protoplasm. We believe that the last named alternative represents the facts, and in support of this hypothesis we now propose to show that the so-called creatine of muscles is a post-mortem product and that there is very little creatine in living muscles." However, their later work, as well as ours, which shows creatine excretion to be induced by liberal protein feeding, would hardly support the statement that creatine at least under certain entirely normal conditions is not a waste product. Benedict and Osterberg<sup>24</sup> have stated: "Creatine is probably being formed in the animal organism in relatively large amounts, and is normally for the most part either utilized or destroyed." Rose and Dimmitt<sup>34</sup> state: "We believe that these data indicate that the theory accepted without direct proof

<sup>33</sup> Folin and Denis, *J. Biol. Chem.*, 1914, xvii, 493.

<sup>34</sup> Rose, W. C., and Dimmitt, F. W., *J. Biol. Chem.*, 1916, xxvi, 345.

by so many investigators, namely, that creatine and creatinine are destroyed in the body, is without foundation in fact."

The difficulty in accepting the hypothesis of creatine destruction lies in the fact that most of the nitrogen of the ingested creatine does not appear in the urine, as Folin<sup>19</sup> observed in the first experiments which led him to consider creatine as a food. Nor have Folin<sup>19</sup> or others<sup>34</sup> found evidence to warrant accepting its transformation into urea or ammonia. There is evidence that in part it may be changed to creatinine, but the larger portion is excreted as unchanged creatine. If it were destroyed we should expect that the urinary constituents, *viz.* ammonia or urea, would be increased, or at any rate that the urinary nitrogen after subtracting creatine and the increased creatinine nitrogen would still show an increase. We can come to only one conclusion and that is that in such instances the creatine was being stored.

That muscle tissue may be exceptionally low in creatine in physiological as well as pathological conditions has been observed by Howe and Hawk,<sup>6</sup> Denis and Kramer,<sup>16</sup> and Baumann and Hines.<sup>35</sup> Acceptance of the storage possibility does not presuppose that an exceptionally high creatine content of muscle need obtain upon the failure of ingested creatine to make its appearance in the urine. Teleologically a more than incidental or moderate accumulation of creatine during creatine administration in the tissues of the body would lead us to be inclined to attribute a specific function to creatine. So far as we are aware, there is no indication that creatine has any specific rôle to perform in the animal body unless it is that by reversible reaction it can serve as a source of the guanidine grouping for arginine synthesis. Such reasoning would lead us too far into the hypothetical without experimental basis to make it worth more than a suggestion for future work.

During short periods of high level ingestion of creatine or creatine precursors, creatine storage might be assumed to occur as the tissues serving for creatine storage might have been originally depleted in creatine; but during long periods of observation where excessive amounts of protein are being fed with no resulting appearance of creatine or increase in creatine excretion the situation cannot be explained so easily. Especially is this true

<sup>35</sup> Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1917, xxxi, 549.

of mature men where there can be no extensive utilization of creatine precursors in tissue construction and therefore of necessity we would infer that the conditions were especially propitious for the formation of large amounts of creatine. Folin and Denis<sup>23</sup> have made the statement that 1 gm. of creatine per day is about the maximum which a full grown man can be made to retain even when kept on low protein diets, and on high protein diets the amounts retained are smaller than on diets low in protein. We are forced to the conclusion if we accept the rest of our theory that either the metabolism of creatine precursors in these individuals proceeds primarily in another direction from that leading to creatine (with arginine this would mean breaking up the guanidine residue) or that the destructive ability of the individual for creatine itself is greater.

By our statements here we hope that we have sufficiently brought out the conditions which our theory of the origin of creatine in the urine would demand so that it may be clearly proved or disproved in other laboratories as well as in our own. It may even be that creatine does not have its origin in the protein metabolism of the exogenous character, but that the metabolism of the ingested protein so stimulates the endogenous metabolism that creatine is produced from it in larger amounts and excreted. With the information at present available we are not inclined to champion this latter theory, but hope later to offer data which will make the precursor theory of the origin of creatine entirely acceptable.

In addition, we desire to present a suggestion in regard to the cause for the difference in the behavior of men and women in the facility with which creatinuria is produced on ingestion of protein. We believe it possible that this may be related to the variations well known to obtain in the activity of the thyroid mechanism. To support this, we have the observation of Denis<sup>20</sup> that in a man afflicted with Graves' disease creatine was observed in the urine even when on a low protein diet. Yet in normal well nourished men so far as we are aware, creatinuria has never been observed or experimentally produced. This led to the early statement that adults did not have creatine in the urine. In women, however, creatinuria usually obtains. Krause<sup>26</sup> at-

<sup>26</sup> Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293.

tempted to relate this to the sexual cycle, but M. S. Rose<sup>25</sup> could not establish such a relation. It is suggestive that periodic variations in thyroid activity occur with the menstrual period varying in degree with different women and that during pregnancy when the thyroid is also affected pronounced creatinuria has often been reported. Such data are too limited and have been collected without regard to consideration of the character of diet, so their value is problematic. In this connection the work of Henderson,<sup>22</sup> previously mentioned, is suggestive in that changes in creatine content of muscle have been observed in tetania parathyreopriva. The relations of the endocrinal secretions to creatinuria might well be investigated.



# SYNTHETIC CAPACITY OF THE MAMMARY GLAND. I.

## CAN THIS GLAND SYNTHESIZE LYSINE?\*

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(Received for publication, September 30, 1918.)

How far special glandular tissues of the body have preserved specific synthetic functions not shown by muscle cells and less active tissue cells is a question concerning which we have little definite knowledge. It is recognized that specialized glands are constantly making products unique in function and very specific in constitution, as for example the enzymes, the ductless gland secretions, etc.; but from how simple a precursor,—simple in chemical structure,—this is accomplished is practically unknown.

The mammary gland synthesizes a special sugar, as for example lactose, but it probably starts with *d*-glucose, which as a precursor offers no unsurmountable difficulties. It makes a variety of fatty acids of special chemical character, but here again, whether originating from carbohydrates or preexisting fats, the chemical steps, at least theoretically, are capable of projecture. This gland makes a special protein, casein, and presumably from amino-acid fragments in the blood. It is also presumed that it makes casein, not through synthesis of amino-acids *de novo*, but by an orderly assembling, in quantitative relations, of those amino-acids found in the casein molecule. Should an amino-acid, occurring in casein and essential for growth, be absent in the diet, would there be a serious interference in casein building? Or does this gland retain special synthetic powers not shown by muscle cells, and in the absence of a specific amino-acid make it from other nitrogenous fragments? If, for example lysine, an amino-acid necessary for growth, is absent from the

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diet, will the mammary gland still make casein and continue a copious flow of milk?

In 1912, Osborne and Mendel<sup>1</sup> published experiments on the rôle of gliadin in nutrition in which they found that with gliadin as the sole source of nitrogen the capacity of the animal to produce healthy young and suitably nourish them was not impaired. At that time gliadin was held to be a protein free from lysine and those experiments left the impression that the mammary gland could synthesize lysine. Later work by Osborne, Van Slyke, Leavenworth, and Vinograd<sup>2</sup> showed that gliadin, purified as far as possible, still contained 1.21 per cent of lysine. In fact, in the first paper by Osborne and Mendel referred to above,<sup>1</sup> the question of the freedom of gliadin from lysine was raised and analysis of the gliadin used showed 0.15 per cent of lysine. In addition to the fact that the gliadin used in the above experiments contained lysine, the use of natural, protein-free milk was practised. While the nature of the nitrogen of the latter material has not been definitely characterized, its efficient supplementing effect, at least for certain materials, has been definitely established.<sup>3</sup>

Therefore, in view of the possible errors creeping into these earlier experiments and the rather important fundamental question at issue, namely the synthetic power of the mammary gland, experiments, initiated 2 years ago for further study of this question, were evolved with zein as the basal protein rather than gliadin. Zein was selected because it is a protein recognized today as free from lysine and tryptophane, and the biological value of this protein harmonizes more fully with its chemical deficiencies than does gliadin.

### EXPERIMENTAL.

Our plan of procedure involved the use of zein diets made complete by properly supplementing with a complete salt mixture, vitamins, tryptophane, and lysine as contrasted with the

<sup>1</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 473.

<sup>2</sup> Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

<sup>3</sup> Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxi, 149.

same diet free from lysine. In some records, because of the relative poverty of lysine in arginine and histidine, both of these amino-acids were added in order that the essential amino-acid content of the ration should be approximately that of casein. The source of water-soluble vitamine in some of our rations was an alcoholic extract of milk powder, while in other rations brewers' yeast was used directly. Our earlier experiments indicated that commercial milk powder was not an abundant source of the water-soluble vitamine. Either this vitamine exists in such stable combination as to resist solution in 95 per cent alcohol, or it has been partly destroyed in the milk-desiccating process. It is also certain that the extraction made with hot 95 per cent alcohol slowly destroys this vitamine.

Tryptophane was prepared in the usual way by pancreatic digestion of casein, and lysine, arginine, and histidine were obtained by acid hydrolysis of casein. Lysine was introduced into the ration as the hydrochloride, while arginine and histidine were introduced as carbonates. Special care was used in the preparation of zein from gluten meal. It was purified by re-solution in alcohol a number of times (three) and finally evaporated on dextrin from an alcoholic solution.

The qualitative composition of the ration was as follows, although somewhat varied in succeeding experiments (see charts). The salt mixture used was the one found in this laboratory as suitable for synthetic rations.

*Composition of Ration.*

	<i>per cent</i>
Zein.....	19.46
Tryptophane.....	0.54
Lactose.....	20.00
Dextrin.....	48.00
Butter fat.....	5.00
Agar.....	2.00
Salts.....	5.00

*Composition of Salts.*

	<i>gm.</i>
NaCl.....	0.173
MgSO <sub>4</sub> (anhydrous).....	0.266
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O.....	0.347
K <sub>2</sub> HPO <sub>4</sub> .....	0.954
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O.....	0.540
Iron citrate.....	0.118
Ca lactate.....	1.300
KI.....	In traces.

In our earlier experiments the dextrin carried an 8 hour, 95 per cent alcoholic extract of 480 gm. of milk powder per kilo of ration as the source of water-soluble vitamine, but as we became suspicious of its inefficiency as a source of this vitamine, dried



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brewers' yeast (0.5 to 2 per cent of the ration) was used as the source of this dietary factor. Preliminary experiments showed that this yeast must be introduced in the proportion of at least 2 per cent of the ration. Less than that amount was insufficient for normal performance.

When lysine was introduced it formed 1 per cent of the ration. In the case of arginine and histidine each was added as 0.5 per cent of the ration.

Vigorous female rats were selected from our rat colony as experimental animals. It was planned to have an animal on the ration some time before it was bred, in order that the effects of the ration would not be obscured by reserve food materials in the digestive tract. In experiments of this character there are always dangers of incorrect interpretation, due to the possible use of reserve tissues for the more special functions of important organs; while this source of error cannot be entirely avoided there should at least be preliminary feeding periods of sufficient length to secure complete replacement of the old diet by the new. However, as our work progressed we were impressed with the great difficulty in securing pregnant animals on lysine-free rations and for that reason preliminary feeding periods were often short. This did not appear to vitiate the results, as those animals on diets free from lysine were invariably unable to rear their young, while a pronounced improvement in the growth and duration of life, although not a perfect rearing, was secured only when lysine was introduced into the diet. We submit these records as evidence that the mammary gland has not the power to synthesize the amino-acid lysine, but must find it preformed in the blood stream.

It appeared possible for the mother rat to build her young, presumably from tissue reserves rather than through synthesis, even in the absence of lysine from the ration. We had a number of animals give birth to apparently normal litters of young (see Charts 1 to 5) on rations free from lysine, but only in a single case was a litter nursed sufficiently to carry it beyond a mere maintenance of life for approximately 2 weeks after birth. In one case part of the litter was nursed for  $3\frac{1}{2}$  weeks and then all succumbed. It is apparent from our results that the amount of nitrogen furnished by 2 per cent of yeast in the ration was too poor in quality and too low in quantity to supplement our lysine-

free ration sufficiently for normal milk flow. We are decidedly of the opinion that what milk was secreted was normal rat's milk and not a milk whose proteins were deficient in lysine. It was in all probability a diminished flow, but a milk of normal composition.

This principle, namely that the composition of the milk produced under distinctly adverse nutritional conditions will be approximately normal in composition, is firmly established wherever it has been possible to apply quantitative analytical methods, as for example in the case of cow's and goat's milk. True, slight variations in milk composition may occur with variations in the diet, but not sufficient to disturb the *nutritive* qualities of the milk.

The work of Palmer and Eckles<sup>4</sup> has definitely established the fact that the mass of the main coloring matter in milk, carotin, is directly dependent upon its supply in the feed ingested. Further, of great interest were the experiments of Andrews<sup>5</sup> in which Philippine women, suffering from beri-beri and who had lost their infant babies, were induced to nurse young pups. These pups developed paralysis of the posterior extremities and exhibited the symptoms characteristic of beri-beri. Because of the fact that this disease is directly related to the deficiency of the water-soluble vitamine in the diet, these experiments were interpreted as indicating that the milk secreted by these women was likewise deficient in this essential.

On this point these experiments, however, are not absolutely conclusive. The rate of growth of the human baby is quite different from that of a pup. Human milk, whenever secreted, may contain the water-soluble vitamine in sufficient quantity to meet the rate of growth of the human infant, but not of a pup. The protein content of human milk is much lower than that of the milk secreted by the bitch, and we should not expect that the former would meet the protein requirements of growing pups. Why should we ignore in experimental inquiries of this kind the firmly established principle of the adaptability of the milk of a species to the young of that species? Failure of cow's milk to meet the complete nutrition of a rat must not be interpreted as a

<sup>4</sup> Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191.

<sup>5</sup> Andrews, V. L., *Philippine J. Sc., B.*, 1912, vii, 67.

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deficient milk for a calf or for a human infant. The rat doubles its weight in 14 days, while it requires 47 days for the calf to double its weight, and 180 days in the case of the human infant.

In harmony with the idea of a vitamine deficiency and conforming to the principle of species adaptability mentioned above, Andrews is of the opinion that the high infant mortality in Manila is due to the deficiency of water-soluble vitamines in the mother's milk. However, no records of flow of milk accompany his discussion. In addition to Andrews' work, that in this laboratory of McCollum<sup>6</sup> and of Steenbock<sup>7</sup> with barley supports the view that both the fat- and water-soluble vitamins of milk are dependent upon their supply from the food and cannot be synthesized by the mammary gland. Therefore, with the exception of coloring matter and probably with the exception of vitamins, although at the present time we are lacking absolutely decisive data on this point, the other organic and inorganic constituents of milk remain practically constant under wide variations of diet.

When the milking animal, through scarcity of structural units, is forced to conserve, it lowers the flow of milk, but maintains the normal composition of the milk. These facts are so well known to investigators of dairy problems that reference to but a single text<sup>8</sup> and to Babcock's work<sup>9</sup> on withholding chlorides from the rations of cows, is necessary to support this view. In Babcock's experiments common salt was withheld from the rations of dairy cows for 5 to 6 months without an appreciable change in the chlorine content of the milk. It contained 0.10 per cent of chlorine at the initiation of the experiment and 0.08 to 0.09 per cent when the critical period was reached. The critical period was that point at which a sharp decline in milk flow set in, with other manifestations of malnutrition. As soon as the chlorides were readministered the tendency was toward a normal flow.

<sup>6</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33.

<sup>7</sup> Steenbock, H., Kent, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxv, 61.

<sup>8</sup> Jordan, W. H., *Feeding of farm animals*, New York, 1917.

<sup>9</sup> Babcock, S. M., *Wisconsin Exp. Station, 22nd Ann. Rep.*, 1905.

These facts, however, must not be confused with the variations in individuals to produce small or large flows of milk on the same rations; nor with the probable ability of cows, with pronounced dairy breeding, to maintain unusual flows of milk of normal composition although receiving wholly inadequate rations as compared with mother rats, where the mammary function has been developed only to supply milk sufficient for the normal growth of the litter.

Where lysine was added to the zein-tryptophane ration not only were normal litters of young born, but in most instances the young were successfully nursed. In no case was the growth of the young at a normal rate, although the litters were reduced to either four or five individuals. In one record (Chart 6) where the source of the water-soluble vitamine was a 95 per cent alcoholic extract of Merrell-Soule skimmed milk powder the behavior of the young indicated a deficiency of this essential in the milk secreted. At the age of 3 weeks they began to be seized with convulsions, loss of muscular control, and thereafter gained little in weight; death terminated their life at the end of 5 weeks.

In other records on the zein-tryptophane-lysine diet, but with the water-soluble vitamine supplied through the introduction of 2 per cent of yeast, no such symptoms of vitamine deficiency developed in the young (see Charts 7 and 8). There was not a normal rate of growth of these young, but a steady, slow growth and an apparently healthy condition of the mother rat and young. The introduction of the lysine had improved the ration to such an extent as to make continued milk secretion possible, while without it the animal was limited merely to the building of the young and an exceedingly scanty flow of milk.

The probable cause of failure to rear the young at a normal rate on a zein-tryptophane-lysine diet was the incomplete absorption of the zein itself. It is not probable that our supply of lysine was inadequate as it constituted 1 per cent of the ration, a quantity five times as large as used by Osborne and Mendel<sup>10</sup> in successful growth experiments with zein. Osborne and Mendel<sup>11</sup> have published data showing the incomplete utilization of

<sup>10</sup> Osborne and Mendel, *J. Biol. Chem.*, 1916, xxv, 1.

<sup>11</sup> Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 177.

zein by rats. While our ration contained approximately 20 per cent of zein, yet if its utilization was reduced 30 to 50 per cent by failure to be absorbed, as in the case of the experiments quoted above, it can readily be understood how milk flow would be greatly reduced. In support of the view that our purified zein ration was partly unsuccessful for rearing the young because of the indigestibility of the zein, we submit a record (Chart 9) showing the successful rearing of the young at normal rates by substituting casein for the zein-tryptophane-lysine combination in the diet. With the same percentage of nitrogen in the ration the casein was much more effective in stimulating and maintaining milk secretion than was the zein-tryptophane-lysine complex. This we attribute to a more complete absorption of the protein, casein.

It was also possible that our zein-lysine ration was limiting milk flow by its paucity in arginine and histidine. These amino-acids are not as abundant in zein as in casein. For that reason there was added to the zein-tryptophane-lysine diet at the rate of 10 gm. per kilo, a mixture of arginine and histidine obtained by the usual separation of these amino-acids from an acid hydrolysis of casein.

The results secured with this ration are shown in Charts 11 and 12. Normal sized litters of young were born and then reduced to four in number. These were nursed by the mother rat and made slow rates of growth considerably below a normal performance. The rate of growth of these young was no greater than on the ration in which the arginine and histidine additions were omitted. Apparently the indigestibility of the zein was counteracting any beneficial effect the arginine and histidine might have had when added to a protein relatively low in these amino-acids but more available.

To satisfy ourselves that the 2 per cent of yeast introduced into the ration would meet the water-soluble vitamine requirement of growing rats a ration was made up with casein as the sole protein, while another ration contained the zein-tryptophane-lysine complex as the carrier of nitrogen. The rats were started at initial weights of 40 to 70 gm. with casein as the sole protein (see Chart 10). Normal growth was secured with 2 per cent of yeast furnishing the water-soluble vitamine. The yeast

used in all our experiments was a dried powder, secured from a Chicago Brewing Company. It was reported to us that in its preparation it had been exposed for a few seconds to a temperature of 176°C.

Since the casein-yeast ration was entirely satisfactory for growth, any deficiency in the zein-tryptophane-lysine ration could, with justice, be assigned to the nitrogen complexes. This deficiency is probably to be attributed mainly to the low digestibility of the zein. Chart 13 shows the growth curve of rats upon the zein-lysine ration containing 2 per cent of yeast. These growth curves were below normal and demonstrate that our ration was still below the possible attainment in synthetic diets containing a more complete and more digestible protein such as casein.

A review of the records secured with our lysine-free ration indicates that in the majority of cases these animals do not even maintain their live weights. Other investigators<sup>12</sup> have published results which indicate that either lysine was dispensable for maintenance or at least not needed to the same degree as, for example, tryptophane. The fact that there is a rapid decline in body weight in the absence of tryptophane, but only a slight decline, or in some cases actual live weight maintenance for periods of many weeks' duration in the absence of lysine, forces one to the conclusion that the above interpretation of their records by Osborne and Mendel and by Geiling was justified. However, in our own records (see Charts 1 to 3) with lysine-free diets there was a steady decline in live weight in two cases, but a phenomenal maintenance of weight in one case (Chart 1) over a period of 24 weeks. These records raise the question, was there really normal maintenance and normal functioning in these animals? We think not. Their coats became rough and soiled, which is always evidence of malnutrition. These rats were extremely nervous, excitable, and old ahead of their time. Estrum periods disappeared, which certainly is not compatible with normal maintenance.

<sup>12</sup> Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325; 1916, xxv, 1. Geiling, E. M. K., *ibid.*, 1917, xxxi, 173.

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Further, in view of the fact that maintenance of live weight is not a sound basis<sup>12</sup> upon which to decide whether or not an animal is in nitrogen equilibrium, we are forced to the conclusion that *normal* maintenance is not possible in the absence of the amino-acid, lysine. In such maintenance records there certainly is the possibility that catabolyzing non-essential tissues can furnish over very long periods of time enough lysine for rehabilitation of those tissues more essential for the maintenance of life. The maintenance of body weight may be either a replacement of loss with water or with fat.

### SUMMARY.

The evidence presented in this paper makes it very probable that the mammary gland has not the capacity to synthesize the amino-acid, lysine.

Further, the evidence submitted supports the view that as far as the proteins are concerned milk secretion, like growth, is ultimately dependent upon the quality and quantity of amino-acids ingested with the food.

Our records also lead us to the view that the amino-acid, lysine, is not dispensable for *normal* maintenance.

<sup>12</sup> Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1918, xxxv, 367.

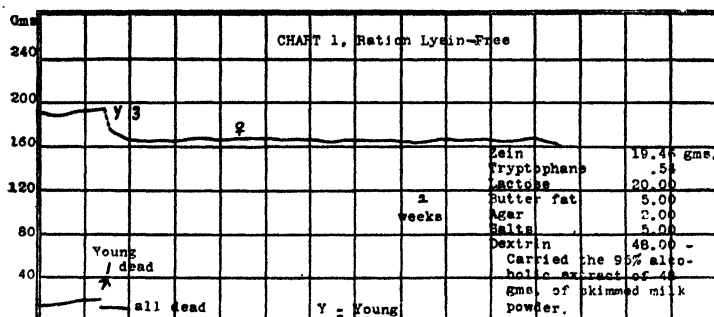


CHART 1. On a lysine-free ration young were born, but not successfully nursed. We believe that this is evidence that the mammary gland has not the power to synthesize lysine. Sacrifice of tissue at a rate sufficient to furnish an adequate supply of milk for milk protein building was not possible. On the other hand, we must in these cases assume that the lysine needed for fetus building came from the catabolized tissue of the mother rat. This rat maintained its live weight on the lysine-free diet, but was rough in appearance and showed no signs of estrum. We doubt if continuous, normal maintenance is possible on a lysine-free diet.

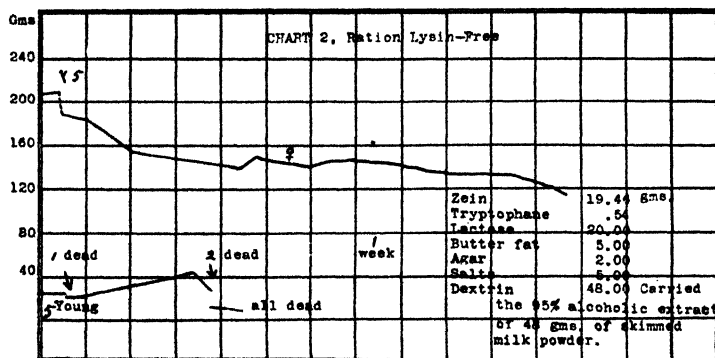


CHART 2. Another failure to rear the young on a lysine-free diet. Milk secretion was probably more abundant in this case than in the case of the record shown in Chart 1, but the rapid loss in live weight would indicate a more rapid tissue metabolism under a stronger impulse for mammary secretion. Further, the shortness of the interval between the time of being placed on the ration and the birth of the young made possible a larger store of lysine-yielding tissue for milk secretion.



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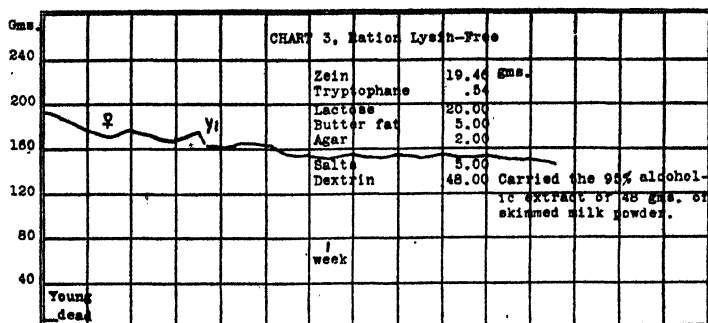


CHART 3. No rearing of the young on a lysine-free ration, but practically a maintenance of live weight after the young were born. Here again the mother rat was rough in coat and far from normal in appearance. We interpret these results as indicating that normal maintenance is impossible without lysine.

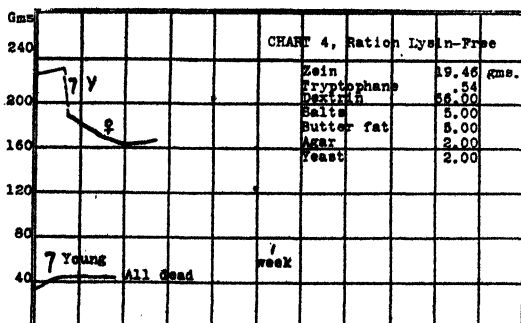


CHART 4. This mother rat, as in previous cases, was wholly unable to nurse her young on a lysine-free ration. The young lived less than 2 weeks, procuring a little milk for maintenance; the lysine, contained in the milk proteins made, undoubtedly was furnished through tissue catabolism by the mother. Even the presence of 2 per cent of yeast in the diet did not furnish nitrogen in quality and quantity sufficient to supplement adequately a zein-tryptophane ration for milk production.

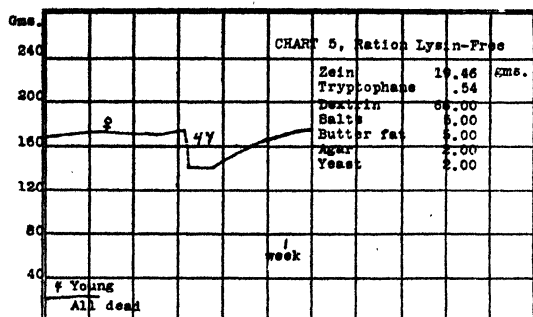


CHART 5. Another failure to rear the young on a lysine-free ration, but containing 2 per cent of yeast as the source of water-soluble vitamin.

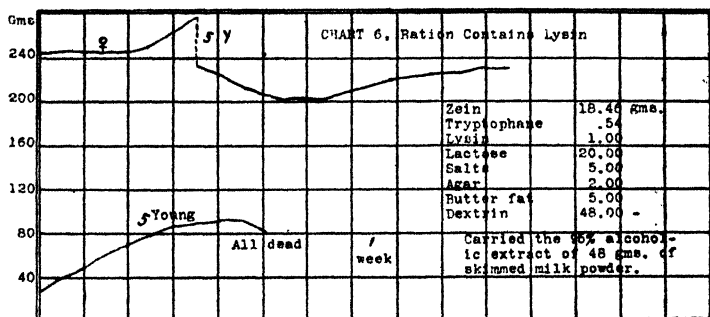


CHART 6. The beneficial effect on milk secretion of lysine in the ration. In the presence of this amino-acid the young were nursed for nearly 5 weeks, at the end of which time there was a sharp decline in weight, and death. 1 kilo of the ration contained the alcoholic extract of 480 gm. of skimmed milk powder. The behavior of the young, characterized by great excitability and impaired locomotion, would indicate a deficiency of the water-soluble vitamin in this ration. That this interpretation of our results is correct is evidenced by the data shown in Charts 7 and 8 where 2 per cent of yeast in the ration was used.

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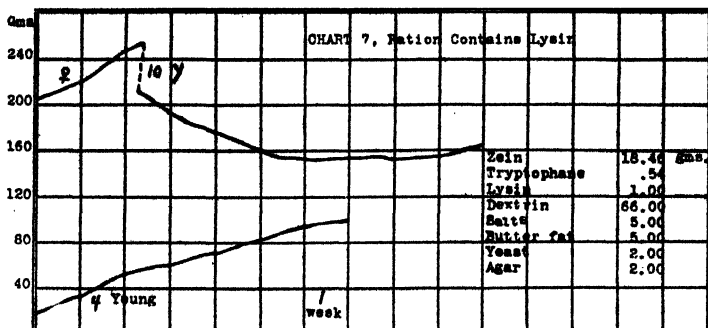


CHART 7. This chart illustrates the effect of both lysine and an adequate supply of water-soluble vitamins furnished by 2 per cent of yeast. This mother rat gave birth to a litter of ten young. We reduced the litter to four in number which were nursed with fair success. Normal rates of growth of the young were not obtained, due no doubt to a diminished milk flow occasioned by the low digestibility of zein. The young, however, were strong and active, but below normal size. For comparison see Charts 4 and 5, which demonstrate that the improvement in the ration was due to the lysine and not to the yeast. With lysine in the ration more copious milk secretion was made possible.

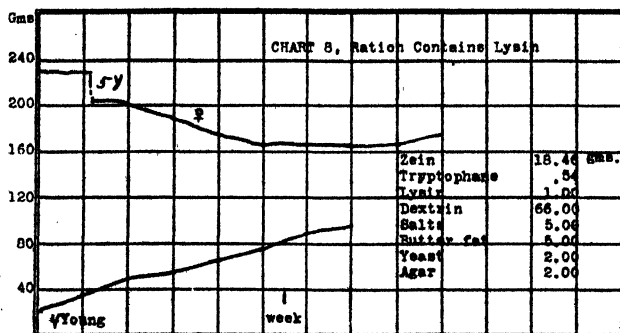


CHART 8. A duplicate of Chart 7 showing the marked effect of lysine in the diet on milk secretion. Without lysine there would have been no rearing of the young. The young grew, however, at a rate below normal, due to the insufficient flow of milk, but were nursed and finally weaned.

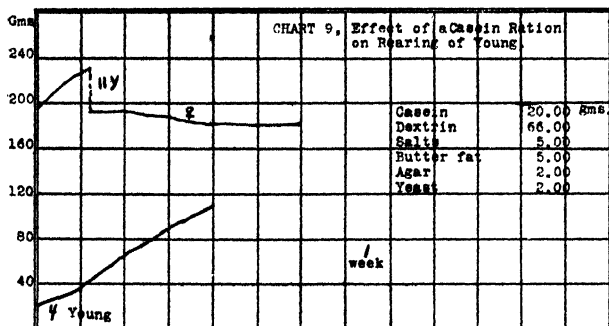


CHART 9. The effect of a good protein on milk secretion. In this ration casein in quantitative equivalence was substituted for the zein-lysine-tryptophane mixture; otherwise the rations were identical. The litter of eleven young was reduced to four in number. The young grew at a much more rapid rate than the litters shown in Charts 7 and 8. With higher efficiency the more completely absorbed casein met the needs of maintenance and a more copious milk secretion than did the less readily digestible protein, zein. This chart further indicates that 2 per cent of yeast could furnish the water-soluble vitamins in quantities sufficient for a nearly perfect rearing of at least four young rats.

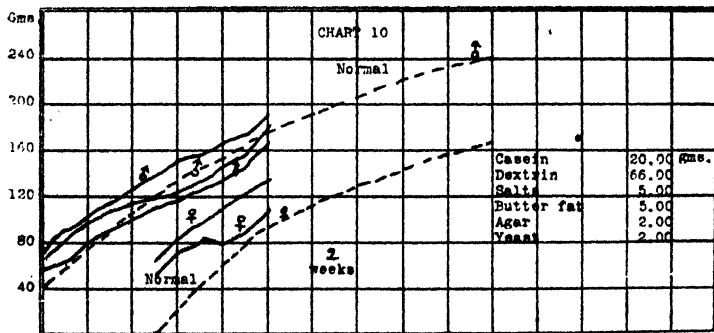


CHART 10. This chart shows that the yeast used, constituting 2 per cent of the ration, was able to furnish sufficient water-soluble vitamins for normal growth of young rats where the other factors of nutrition were adequately provided. These animals were in excellent condition.

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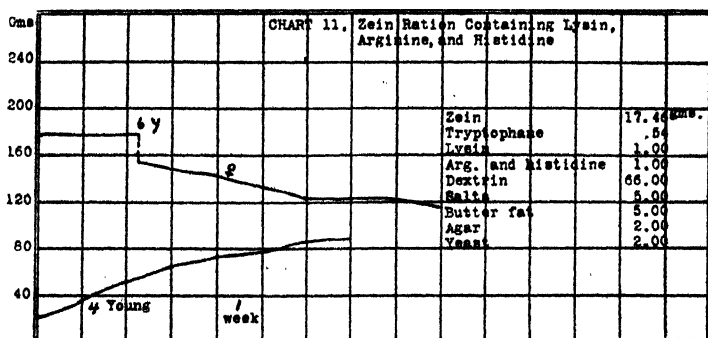


CHART 11. This chart illustrates the effect on milk secretion of adding arginine and histidine to a zein-lysine-tryptophane ration. The addition did not improve the ration for milk secretion. The young made no better growth than those whose mother received a zein-tryptophane diet fortified only with lysine. The indigestibility of the zein was the principal factor depressing the efficiency of this ration for milk secretion.

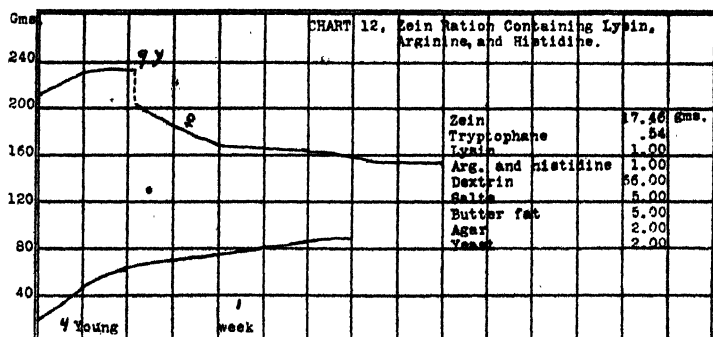


CHART 12. A duplicate of Chart 11, showing the failure of additions of arginine and histidine to a zein-lysine-tryptophane ration to make this nitrogen complex equal in nutritive value to that of an equivalent amount of casein. While the young were nursed and reared to the time that they could be weaned, yet their rate of growth was not a normal one.

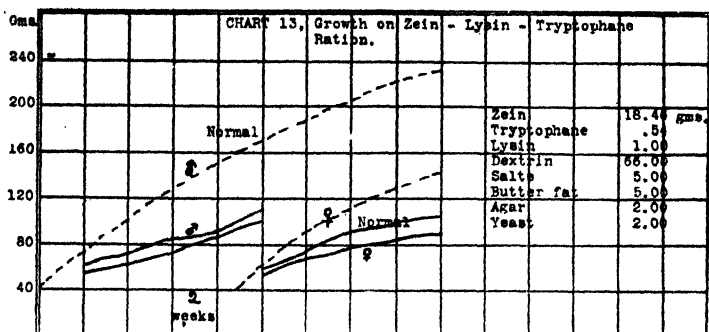


CHART 13. The curves of growth of young rats on the zein-lysine-tryptophane ration. Started at 50 to 60 gm. in weight, nevertheless they were unable to make normal growth on this ration. Compare with Chart 10 where casein, as the sole protein and supplemented in reference to the other nutritive factors in identically the same manner as was the zein-lysine complex, served to support normal growth.



## COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS. II.

### FATE OF *p*-HYDROXYLBENZOIC ACID AND *p*-HYDROXYLPHENYL-ACETIC ACID IN THE ORGANISM OF THE MONKEY.

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(Received for publication, September 23, 1918.)

It has previously been shown that phenylacetic acid in the organism of the monkey undergoes the same process of metabolism as in the lower animals, and is subsequently excreted in combination with glycocoll as phenaceturic acid (1). On the other hand, if ingested by man it undergoes apparently an entirely different process of metabolism, being excreted in the urine in combination with the amino-acid glutamine (2) and known as phenylacetyl glutamine. From a biological standpoint it is of interest to know how far the metabolism of the monkey belongs to that of the lower animals or to that of man in those points where a difference has been demonstrated. Other investigators have shown that the monkey follows the course of the lower animals in the purine metabolism (3) as well as in the ratio of ethereal sulfates to inorganic sulfates (4) excreted in the urine. These isolated facts have little or no value until we have secured enough data to correlate and in this way to follow the fate of each amino-acid during the entire process of metabolism. *p*-Hydroxybenzoic acid and *p*-hydroxyphenylacetic acid are two members of a series of compounds which result from the action of putrefactive bacteria on the aromatic amino-acids. These compounds, all of which are more or less toxic, are quickly detoxicated and rendered sufficiently soluble for their elimination in the urine. The method of detoxication is usually by conjugation with the sulfate radical, glycuronic acid, one of the shorter amino-acids, such as glycocoll, cystine, or glutamine, or



even at times with urea. Authors do not entirely agree as to the fate of the aromatic hydroxyl acids or as to the method of their elimination. One fact seems to be universally accepted, that *p*-hydroxylbenzoic and *p*-hydroxylphenylacetic acids are among the least toxic of all the putrefactive products thus formed.

The former acid when fed to dogs was found in the urine as such by E. Salkowski and H. Salkowski (5) with an augmentation of the quantity of ethereal sulfates, indicating as they believed, a conjugation with the sulfate radical. Baumann and Herter (6) found a decided increase in ethereal sulfates after administering a dose of 2 gm. to a dog but failed to find this same condition after administering an equivalent amount to a human being. They recovered most of the acid from the dog's urine unchanged but found also a trace of *o*-hydroxylhippuric acid. Bertagnini (7) claims to have found relatively large amounts of *o*-hydroxylhippuric acid after animal feeding with the *o*-hydroxylbenzoic acid. Maly (8) after ingesting the acid himself, finds a compound in the urine which he believed to be a conjugation of *p*-hydroxylbenzoic with ethyl or methyl glycocholate, but this, as Schotten (9) explained, was on account of error in analysis, and the compound recovered from the urine was undoubtedly *p*-hydroxylhippuric acid. Schotten ingested 26 gm. of *p*-hydroxylbenzoic acid in the course of 28 hours. He recovered 13.432 gm. (51.66 per cent) from the urine. Of this 9.182 gm. (35.32 per cent) occurred as uncombined *p*-hydroxylbenzoic acid, while 6.004 gm. of the combination with glycocholate was excreted as *p*-hydroxylhippuric acid, which contained 4.25 gm. of *p*-hydroxylbenzoic acid (16.34 per cent). *p*-Hydroxylphenylacetic acid occurs normally in both human urine and in the urine of animals in small amounts. Baumann (10) evaporated 25 liters of normal human urine and isolated from this approximately 0.5 gm. of hydroxylaromatic acids, consisting mostly of *p*-hydroxylphenylpropionic and *p*-hydroxylphenylacetic acids. Again (11), from 240 liters of human urine he isolated 4 gm. of impure *p*-hydroxylphenylacetic acid and a small amount of *p*-hydroxylphenylpropionic acid. These acids he believed to be excreted mostly as the sodium salts and perhaps partly as ethereal sulfates which might have been destroyed in the process of their isolation. Salkowski and Salkowski (12) after feeding 2.5 gm. of *p*-hydroxylphenylacetic acid to a dog, found most of the acid unchanged in the urine but found also a small amount of a substance which came out of solution as "krystallwarzen" (wart-like crystals) in rather an impure condition and melted at 153°. The amount was too small for an analysis but they hydrolyzed a small portion of it by boiling with HCl and were able to isolate *p*-hydroxylphenylacetic acid from the acid solution. They concluded their compound was *p*-hydroxylphenylacetic acid. They do not mention the exact amount of the acid which they isolated from the urine. A repetition of this experiment failed to yield even a trace of the acid. The same dog which had excreted a small

amount of the acid after a 2.5 gm. dose of the *p*-hydroxylphenylacetic acid failed to conjugate any of this acid after a 6 gm. dose and excreted the *p*-hydroxylphenylacetic acid unchanged in the urine. Experiments on other dogs and even on rabbits also met with failure. A rabbit received 2 gm. of *p*-hydroxylphenylacetic acid and excreted 1.25 gm. of the acid in the urine. Schotten (9) ingested 7.5 gm. of the acid and noticed that the urine collected only a short time after gave a strong coloration with Millon's reagent. This reaction continued with decreasing intensity until 10 hours after the ingestion of the acid. From the urine he regained 5.90 gm. (78.6 per cent) of the acid unchanged but found no trace of *p*-hydroxylphenaceturic acid.

In this laboratory a monkey was fed *p*-hydroxylbenzoic acid and *p*-hydroxylphenylacetic acid.<sup>1</sup> The *p*-hydroxylbenzoic acid was in each case excreted in the urine and no combination with glycocholic acid could be found. The amounts regained were 50, 56.03, and 60.07 per cent of 1, 2, and 3 gm. doses, respectively. Most of the *p*-hydroxylphenylacetic acid also was excreted in the urine unchanged but in one instance 0.78 gm. of *p*-hydroxylphenaceturic acid was found in the urine after a feeding of 2 gm. of the acid, followed 24 hours after by a 2.5 gm. dose. The *p*-hydroxylphenaceturic acid was purified, analyzed, and studied.

#### EXPERIMENTAL.

A female monkey (*Macacus rhesus*) of 4.2 kilos body weight, was placed on a regular diet of milk, bread, bananas, and apples for several days to determine as nearly as possible the amount of aromatic hydroxyl acids in the urine. The amount of these acids was found to be negligible. The monkey was placed in a small metabolism cage and the urine collected for a period of 36 hours after administering the acid. The acid was given in different sized doses but the entire quantity was given at one time and not throughout a period of several hours. In amounts not exceeding 1 gm. the acid was given as a solution of the sodium salt in milk. In larger quantities a water solution of the sodium salt was introduced directly into the stomach by means of a stomach tube. The different portions of urine excreted during the first 36 hours after the acid feeding were united, carefully

<sup>1</sup> The *p*-hydroxylphenylacetic acid used for this work was prepared by Dr. W. A. Jacobs of The Rockefeller Institute.

neutralized with sodium carbonate, and slowly evaporated on a water bath at low temperature. In most cases the urine after reaching a thick syrupy consistency was cooled, acidified with phosphoric acid until it gave a distinct reaction with Congo red, and then extracted several times for a period of 2 or 3 hours with ether, alcohol, and ethyl acetate in a continuous extracting apparatus. By this means it was possible to make a rough separation between the *p*-hydroxylbenzoic acid and the *p*-hydroxylhippuric in case the latter should be present, and again between the *p*-hydroxylphenylacetic and the *p*-hydroxylphenaceturic acids. In both cases the free acids are much more soluble in ether than their corresponding glycocoll compounds. After extraction, the extract was evaporated to dryness by stages so there was opportunity for crystals to appear at the different concentrations. The residue left was dissolved in warm water and the water solution concentrated at regular intervals during a period of several days. Crystals which appeared were purified by dissolving in water, boiling with charcoal, and again recrystallizing from water. In each case the mother liquor from crystals or the residue from the extraction (if no crystals appeared) was boiled with a 35 to 40 per cent solution of  $H_2SO_4$ , cooled, and extracted with ether. By this method it was possible to tell whether any of the hydroxyl acids in a conjugated form had failed to crystallize out of the water solution.

*Experiment I with p-Hydroxylbenzoic Acid.*—1 gm. of *p*-hydroxylbenzoic acid was fed to the monkey as a solution of the sodium salt in milk. There were no noticeable physiological effects as loss of appetite, dizziness, signs of nausea, or even increased thirst. This seemed to show that the acid is almost non-toxic. The urine for the first 36 hours after the feeding amounted to 130 cc. The general method of extracting was followed. The evaporated urine was extracted three times with ether. Each period of extraction covered 3 hours and about 300 cc. of ether were used for each extraction. All ether was then removed from the extracting apparatus and the urine was extracted for a period of 3 hours with the same amount of alcohol. Again the urine was extracted twice with ethyl acetate. Each extraction lasted about 3 hours and 300 to 350 cc. of ethyl acetate were used. Each of the three ether extracts was evaporated separately; no crystals appeared, so the residue from each was dissolved in hot water, boiled with charcoal, and filtered. After a few hours standing, crystals appeared in the water solution of the first ether extract but none appeared in the water solution of either the sec-

ond or third extract. The water solutions of the three ether extracts were each boiled with acid, cooled, and extracted with ether but no *p*-hydroxylbenzoic acid could be found except the merest trace which probably had failed to crystallize out of the water solution. The alcoholic and ethyl acetate solutions contained small amounts of hippuric acid and much urea which had been carried over mechanically, but even after boiling the residues with acid no measurable amount of *p*-hydroxylbenzoic acid could be found. The crystals from the first ether extract were *p*-hydroxylbenzoic acid, as is shown below. The substance was twice recrystallized from water and dried at 100°; melting point 208–210.°

#### Analysis.

0.1837 gm. gave 0.4094 gm. CO<sub>2</sub>. 0.0729 gm. H<sub>2</sub>O.

	Calculated for C <sub>11</sub> H <sub>8</sub> O <sub>3</sub> :	Found:
C.....	60.86	60.79
H.....	4.40	4.44

*Experiment II with p-Hydroxylbenzoic Acid.*—After a few days the monkey was given a dose of 2 gm. of *p*-hydroxylbenzoic acid, followed in a few days by a dose of 3 gm. The water solution of the residue from the ether extracts yielded in the first case 1.127 gm. of the free *p*-hydroxylbenzoic acid, and in the latter case 1.822 gm. The mother liquor from these crystals yielded no *p*-hydroxyhippuric acid nor was more than a trace of *p*-hydroxylbenzoic acid extracted with ether after the boiling of this liquid with 40 per cent H<sub>2</sub>SO<sub>4</sub>. The alcohol and ethyl acetate extracts of these urines yielded only small amounts of *p*-hydroxylbenzoic acid.

#### Discussion of Experiments with *p*-Hydroxylbenzoic Acid.

The results obtained from the feeding of this acid are summed up in Table I, which shows that from 50 to 60 per cent of the acid

TABLE I.

	Amount fed.	Amount extracted from urine.	
	gm.	gm.	per cent
<i>p</i> -Hydroxylbenzoic acid.....	1	0.5	50
“ “ .....	2	1.127	56.03
“ “ .....	3	1.802	60.01

fed was recovered from the urine in a free state with the formation of no perceptible amount of *p*-hydroxyhippuric acid. The acid is probably excreted as the sodium salt and perhaps in very small amounts as ethereal sulfate. Baumann and Herter

(6) believed that the sulfate compound existed in the urine but that it was destroyed during the experimental work. This compound of the acid, later prepared synthetically by Baumann (13), proved to be very stable and quite insoluble in organic solvents. It is possible that small amounts of the acid might have been present in the urine as a glycuronic acid compound analogous to that found by Magnus-Levy (14) in the urine of sheep after feeding benzoic acid. The amount of this compound present must have been small, as the optical rotation of the urine before and after the feeding was practically the same.

*p*-Hydroxyphenylacetic Acid.

The monkey was able to stand as much as 3 gm. of the *p*-hydroxybenzoic acid (0.685 gm. per kilo of body weight) without any apparent signs of discomfort, but 2.5 gm. of *p*-hydroxyphenylacetic acid seemed to be the maximum dose which the animal could comfortably tolerate. The acid appeared to cause considerable thirst and corresponding higher urine volumes than after *p*-hydroxybenzoic acid feeding.

*Experiment I with p-Hydroxyphenylacetic Acid.*—1 gm. of *p*-hydroxyphenylacetic acid was fed to the monkey as a solution of the sodium salt in milk. The urine volume collected the first 36 hours was 186 cc. The urine was evaporated to a small volume, made acid, and was shaken repeatedly with ether in a separatory funnel. About 350 cc. of ether were used for each extraction. The different ether extracts were united and the ether was distilled off. The residue was dissolved in hot water, boiled with charcoal, and filtered. The filtrate was neutralized with  $\text{Na}_2\text{CO}_3$  and shaken with a large volume of ether to remove pigment matter and other ether-soluble impurities. The water solution was acidified and again extracted with ether. This ether extract, when evaporated left a mass of crystalline material. This material was twice recrystallized from water and dried at  $100^\circ$ . The melting point was between  $146.5$  and  $148^\circ$  and the substance was therefore relatively pure *p*-hydroxyphenylacetic acid. The urine was next shaken repeatedly in a separatory funnel with large quantities of ethyl acetate. The different portions of ethyl acetate were united and slowly evaporated to dryness. The residue consisted mostly of urea and hippuric acid. The entire mass was dissolved in the smallest possible amount of hot water and left for 24 hours. In this way a few crystals of hippuric acid (melting point,  $187^\circ$ ) were removed from the liquid. The entire solution was boiled with an equal volume of 40 per cent  $\text{H}_2\text{SO}_4$  for 1 hour, cooled, and extracted several times with ether.

As no *p*-hydroxyphenylacetic acid was found in the ether extract it seemed certain that neither the free nor combined acid had previously existed in the ethyl acetate extract of the urine. The entire amount of *p*-hydroxyphenylacetic acid found in the ether extract of the urine was 0.32 gm.

*Experiment II with p-Hydroxyphenylacetic Acid.*—2 gm. of *p*-hydroxyphenylacetic acid fed to the monkey were followed after 24 hours by a dose of 2.5 gm. The urine was collected for 36 hours after the second dose or for a total period of 60 hours. The entire urine volume was 420 cc., and was strongly acid to litmus. The urine was handled in the usual way but was extracted immediately with ethyl acetate. The evaporated urine for the 60 hour period was extracted three times for periods of 3 hours each with 300 cc. of ethyl acetate in the continuous extracting apparatus. These extracts will be called Nos. 1, 2, and 3. Each day about one-third of the remaining volume of each extract was distilled off and then placed in the ice box during the interval. Quantities of urea crystallized out of each extract but especially out of No. 3. Small amounts of hippuric acid crystallized out of Extracts 1 and 2 as they approached the point of dryness. The residue from Extract 1 was found to be of definite crystalline structure but apparently very impure. The residue from Extract 2 contained the least amount of material, and No. 3 the greatest. These three residues were dissolved in the least possible amount of hot water and neutralized with  $\text{Na}_2\text{CO}_3$ . The water solutions were shaken with ether to remove coloring matter and impurities, then acidified, and again shaken with ether. The last ether extracts were evaporated, the residue was dissolved in water, boiled with charcoal, filtered, and the filtrate allowed to stand in the cold. From the water solutions of Extracts 1 and 2 quantities of *p*-hydroxyphenylacetic acid were regained, amounting in all to 2.177 gm.

The water solutions of the residue from each of the three original ethylacetate extracts had thus far yielded only the uncombined acid. In order to recover any of the combined acid still held in the solutions, each water solution of the ethyl acetate residue was shaken many times with ethyl acetate. These ethyl acetate extracts were evaporated and the small amount of material remaining was taken up in hot water. No crystals appeared until the solution was evaporated almost to dryness, when groups of fungus-like masses appeared on the sides and bottom of the crystallizing dish; this was especially so in the case of Extract 1 and to a slight degree in Extract 2. These fungus-like masses were found to consist of groups of minute needles. These groups of crystals were collected and recrystallized from water. When recrystallized from not too concentrated solution they appeared in the form of thin hexagonal plates. In all, 0.78 gm. of this substance was isolated from the urine. The melting point of the substance when dried at 95–100° is 154.5–155°. It is relatively soluble in alcohol, ethyl acetate, and warm water, but quite insoluble in ether, benzene, and cold water. A small amount of the substance was boiled with concentrated HCl for 1 hour, the acid solution was cooled, and extracted several times with ether. The residue from the ether extraction when

recrystallized from water proved by its melting point to be *p*-hydroxylphenylacetic acid. The melting point was 147–148°. When boiled with copper oxide a blue solution of the copper salt results. The copper salt is extremely soluble in water and crystallizes in irregular crystals only when the solution is evaporated nearly to dryness. The compound isolated from the ethyl acetate extract is undoubtedly the glycocoll combination of *p*-hydroxylphenylacetic acid (*p*-hydroxylphenaceturic acid).

#### Analysis.

a. 0.1021 gm. substance gave 0.2145 gm. carbon dioxide and 0.0488 gm. water.

b. 0.1127 gm. substance gave 0.2370 gm. carbon dioxide and 0.0541 gm. water.

c. 0.1055 gm. substance required 5.05 cc. 0.1 N hydrochloric acid.

	Calculated (Kjeldahl) for $C_{10}H_{11}NO_4$	Found:	
	per cent	per cent	per cent
C.....	57.39	a. 57.30	b. 57.35
H.....	5.30	a. 5.34	b. 5.38
N.....	6.68	c. 6.70	

#### Discussion of Experiments with *p*-Hydroxylphenylacetic Acid.

In Experiment I, after feeding 1 gm. of *p*-hydroxylphenylacetic acid, 0.32 gm. (32 per cent) of the acid was recovered from the urine. All of this acid was in the uncombined state and none of the glycocoll combination could be found. In Experiment II, after feeding a total of 4.5 gm., 2.177 gm. were recovered from the urine as the uncombined acid as well as 0.78 gm. of *p*-hydroxylphenaceturic acid. A total of 2.684 gm. of the *p*-hydroxylphenylacetic acid, or 59.64 per cent, was regained after feeding, as compared to 78.66 per cent recovered by Schotten (9) from his own urine after he had ingested 7.5 gm. of the acid. Of this 59.64 per cent isolated from the urine, 48.37 per cent was in the form of uncombined acid and only 11.27 per cent in the form of the glycocoll compound. The failure to find some of the conjugated acid after feeding of the 1 gm. is in contrast to the work of Salkowski and Salkowski (12) who found a small amount of the acid after 2.5 gm. feeding but none at all after feeding the same dog a dose of 6 gm. That the animal organism is able to

furnish much more glycocoll than the amount necessary to combine with even 6 gm. of the acid has been shown by the numerous experiments reported after the feeding of benzoic acid. It seems most probable that both of these acids, the *p*-hydroxybenzoic acid and *p*-hydroxyphenylacetic acid, are at least to a large degree combined with glycocoll or some other amino-acid as a first step in the process of their elimination. This compound then undergoes a more or less complete oxidation depending on the metabolic process common to a given species of animal. In this there may be the formation of the glycocoll compound from almost any of the amino-acids as well as a partial destruction of the glycocoll compound and elimination of some of the free aromatic acid. Magnus-Levy (15) has shown that benzoylated amino-acids are not changed into hippuric acid in the animal organism but are excreted in the same form in the urine. Thierfelder and Sherwin on the contrary find phenaceturic acid in the urine of a dog after feeding phenylacetyl glutamine.

#### CONCLUSION.

1. After feeding a monkey *p*-hydroxybenzoic acid 50 to 60 per cent of it was isolated from the urine as the free acid.

2. When a monkey received 1 gm. of *p*-hydroxyphenylacetic acid 32 per cent of it was found in the urine in an uncombined state. After feeding 4.5 gm., 48.37 per cent was regained from the urine in an uncombined state while 11.27 per cent occurred (in combination with glycocoll) as *p*-hydroxyphenaceturic acid.

3. *p*-Hydroxyphenaceturic acid, found in only a very small amount by Salkowski and Salkowski, was obtained in sufficient amounts for analysis and the preparation of the copper salt.

4. The process of metabolism of the aromatic amino-acids in the monkey is the same as that of the lower animals and different from that of man. The monkey excretes the *p*-hydroxybenzoic acid in the urine in an uncombined state while a partial combination with glycocoll takes place in the human organism. The *p*-hydroxyphenylacetic acid is partly excreted as *p*-hydroxyphenaceturic acid in the case of the monkey and lower animals but is excreted in an uncombined form in the urine of man.



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## THE DETERMINATION OF TYROSINE IN PROTEINS.

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(Received for publication, September 24, 1918.)

In the determination of the amino-acids in proteins, the tyrosine is usually estimated by hydrolyzing a separate 50 gm. portion of the protein in addition to the larger quantity used for the esterification. The hydrolysis may be accomplished by means of either sulfuric or hydrochloric acid. In most instances sulfuric acid has been employed. After the hydrolysis the sulfuric acid is removed quantitatively by means of barium hydroxide. This results in a large precipitate of barium sulfate which must be washed repeatedly to remove all of the tyrosine. The tyrosine, together with the other amino-acids, is thus left in a large volume of water which must be concentrated before the tyrosine will crystallize out.

When hydrochloric acid is used the hydrolysate is filtered to remove humin and the filtrate is decolorized with carbon. It is then concentrated under diminished pressure to a sirup in order to remove as much hydrochloric acid as possible. The residue is diluted to 1 liter with water. The chlorine content is determined in an aliquot part and hydrochloric acid is neutralized by adding the calculated quantity of potassium hydroxide. The final volume of solution thus obtained is less than when sulfuric acid is used and there is no precipitate to adsorb the tyrosine. This method is therefore the more convenient and less laborious.

In a recent hydrolysis of kafirin, the alcohol-soluble protein of kafir, *Andropogon sorghum* (1), determinations of tyrosine were made by both methods and the quantities found were 2.48 and 2.45 per cent, respectively. In both determinations the solutions were gradually concentrated with the removal of successive crops of tyrosine until only traces of tyrosine crystallized out, together

with considerable quantities of other amino-acids. Nevertheless, the mother liquors still gave tests for tyrosine with Millon's reagent showing that all of the tyrosine had not been isolated.

A third determination was therefore made using the colorimetric method of Folin and Denis (2) after hydrolyzing 1 gm. of kafirin by boiling for 12 hours with 25 cc. of 20 per cent hydrochloric acid. This determination indicated the presence of 5.49 per cent of tyrosine in kafirin.

When tyrosine is to be isolated from a protein it is customary to boil the protein with the mineral acid for from 18 to 30 hours or even longer. It is questionable whether it is advisable to boil for so long a period. Gortner (3) has shown that under certain conditions tyrosine is slowly decomposed when a protein is boiled with hydrochloric acid. In order to ascertain whether more tyrosine can be isolated by decreasing the duration of the hydrolysis, a 50 gm. sample of kafirin was hydrolyzed for 12 hours with 20 per cent hydrochloric acid. In this experiment 3.91 per cent of tyrosine was isolated. This is 1.43 per cent more than was obtained when kafirin was hydrolyzed for 48 hours. It therefore seems unnecessary and even detrimental to continue the hydrolysis for more than 12 hours. It is very unlikely that all of the tyrosine was isolated in this experiment since it is practically impossible to crystallize out all of the tyrosine from the hydrolysates of most proteins. It is therefore probable that the percentage of tyrosine indicated by the colorimetric method of Folin and Denis represents more accurately the quantity of tyrosine present in the protein than does the percentage obtained by direct isolation.

Objections have been raised by Abderhalden and Fuchs (4) and Abderhalden (5) to the colorimetric method of Folin and Denis on the ground that tryptophane, oxytryptophane, and *l*-oxyproline, if present, will cause high results. These authors, however, report only qualitative tests with the above amino-acids and do not consider the effect of hydrolysis. It is true that tryptophane gives a blue color with the reagent of Folin and Denis, but the intensity of the color is much less than that given by an equivalent weight of tyrosine. It is well known that tryptophane is decomposed by acid hydrolysis. To ascertain whether this decomposition is complete and that the decomposition products do not give a color with the reagent of Folin and

Denis, the following experiment was made: 5 per cent of tryptophane was added to 0.5 gm. of kafirin and the mixture was boiled with 20 per cent hydrochloric acid for 12 hours. The tyrosine in the hydrolysate was estimated by the method of Folin and Denis and was found to be 4.36 per cent. A hydrolysis of kafirin performed under similar conditions without the addition of tryptophane gave 4.84 per cent of tyrosine. Hence the tryptophane was completely decomposed and its decomposition products gave no blue color with the reagent of Folin and Denis. It is also to be expected that oxytryptophane, if present, would be decomposed by acid hydrolysis, since the presence of the hydroxyl group would probably render it less stable than tryptophane. A sample of gelatin to which tryptophane had been added was also hydrolyzed. The blue color obtained with the reagent of Folin and Denis was of the same intensity as that obtained by a control hydrolysis where no tryptophane was added. The faint blue color obtained from the hydrolysate of the gelatin was probably due to tyrosine since the gelatin gave a distinct test for tyrosine with Millon's reagent.

Abderhalden (5) states that *l*-oxyproline gives a blue color with the reagent of Folin and Denis. On the other hand, Folin and Denis (2) obtained only a faint blue color from the hydrolysate of gelatin which contains 3 to 6 (6) per cent of oxyproline. This color was probably due to tyrosine. We tested a number of high grade samples of gelatin and did not find one that did not respond to the test for tyrosine with Millon's reagent. Even gelatin prepared from carefully cleaned cartilaginous rings of ox trachea gave a decided test for tyrosine.

Tyrosine and cystine are the least soluble in water of all the amino-acids obtained from the hydrolysate of a protein. Most of the cystine present in proteins is decomposed by prolonged acid hydrolysis and does not interfere with the isolation of tyrosine from most proteins. On the other hand, the presence of considerable quantities of other amino-acids in a hydrolysate may render the tyrosine readily soluble. We encountered a case in which considerable tyrosine was separated from a hydrolysate. After this tyrosine had been removed a large crop of almost pure leucine separated. On concentrating the 'mother liquor' considerably more tyrosine mixed with a little leucine was obtained. In an-

other instance we had a similar experience with a fraction of a hydrolysate from which the leucine and some valine had been removed and which on further concentration still yielded some pure tyrosine.

#### CONCLUSIONS.

The method of Folin and Denis for the determination of tyrosine has been investigated. It has been found that tryptophane is completely decomposed during the hydrolysis of proteins with hydrochloric acid and that the decomposition products do not interfere with the determination of tyrosine by the method of Folin and Denis. Since tyrosine is decomposed to some extent during hydrolysis there seems to be no advantage in hydrolyzing more than 12 hours. It has been shown that oxyproline does not interfere with the determination of tyrosine by the method of Folin and Denis. Gelatin which is said to contain up to 6 per cent of oxyproline gave but little color with the reagent of Folin and Denis after hydrolysis. This color was probably due to tyrosine in the gelatin since a test for tyrosine was obtained by Millon's reagent.

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## THE HYDROLYSIS OF KAFIRIN.

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(Received for publication, September 24, 1918.)

Kafirin is the alcohol-soluble protein of kafir, *Andropogon sorghum*. The sample used in this investigation was prepared according to the method previously published (1). The methods employed in the hydrolysis of kafirin are essentially those devised by Fischer and modified by Osborne. Certain modifications recommended by other investigators were found helpful and are noted below.

The esterification of the amino-acids was accomplished by the method of Phelps and Phelps (2, 3). The esters were liberated from their hydrochlorides with sodium ethylate instead of sodium hydroxide. This change was suggested by Abderhalden and Rostoski (4). Instead of distilling the esters directly after filtering off sodium chloride and evaporating the alcohol, the esters were mixed with water and extracted with ether. This made it possible to make a second esterification of the portion not soluble in ether. Since a 3 per cent solution of sodium ethylate in alcohol was used to liberate the esters from their hydrochlorides, the free esters were left dissolved in a large volume of alcohol. When this was distilled off, some of the esters were carried over. These were recovered by adding hydrochloric acid to the receiver and finally evaporating the alcohol. The ether with which the esters had been extracted also carried over esters equivalent to 12 gm. of amino-acids. These were recovered by acidifying the ether with hydrochloric acid and evaporating the ether after the mixture had stood for some weeks to allow any glycine hydrochloride that might be present to separate. Osborne and Liddle (5) have shown that other esters as well as that of glycine can be carried over with the ether.

Tyrosine was first determined in the usual manner by making separate hydrolyses of 50 gm. samples of the protein. Two determinations were made, using sulfuric acid for one hydrolysis and hydrochloric (6) acid for another. These methods gave similar results; namely, 2.45 and 2.48 per cent, respectively. Since it is practically impossible to crystallize all of the tyrosine from a solution of the hydrolysate of a protein, it seemed probable that these results were low. A third determination of tyrosine was therefore made, using the colorimetric method of Folin and Denis (7, 8). This determination indicated the presence of 5.49 per cent of tyrosine in kafirin, or more than twice as much as was obtained by direct crystallization in the first two determinations. A fourth determination of tyrosine was made by hydrolyzing the kafirin with hydrochloric acid for only 12 hours and crystallizing in the usual manner. This determination gave 3.91 per cent of pure tyrosine.

The determination of aspartic acid was made by the method of Foreman (9) on a separate 50 gm. portion of the protein. Much higher results were obtained by this method than by separating the aspartic acid from the esters.

The method of Van Slyke and Levene (10) for the separation of leucine and valine was employed and found to be very satisfactory.

The percentages of the basic amino-acids in kafirin, as determined by the Van Slyke method have already been published (1). Two new determinations were made by Mr. A. J. Finks of this Laboratory, and they agreed well with the previous results. The percentages of the different amino-acids obtained in the hydrolysis of kafirin have been summarized in Table I. The values recorded in this table for leucine, proline, and glutaminic acid are somewhat higher than the figures given in the experimental work here recorded. They were obtained by a method of isolation which is now under investigation in this Laboratory. Since kafirin resembles zein, the alcohol-soluble protein of maize, in many respects, the percentages of amino-acid found in zein are also given for the sake of comparison. The values given for zein are compiled from the results of several hydrolyses and represent the highest reliable values recorded.

TABLE I.  
*Percentage of Amino-Acids in Kafirin and Zein.\**

	Kafirin.	Zein.*
	<i>per cent</i>	<i>per cent</i>
Glycine.....	0.00	0.00
Alanine.....	8.08	9.79
Valine.....	4.26	1.88
Leucine.....	15.44	19.55
Proline.....	7.80	9.04
Phenylalanine.....	2.34	6.55
Aspartic acid.....	2.27	1.71
Glutaminic acid.....	21.23	26.17
Serine.....	—	1.02
Oxyproline.....	—	—
Tyrosine.....	5.49	3.55
Cystine.....	0.84	—
Arginine.....	1.59	1.55
Histidine.....	1.12	0.82
Lysine.....	0.95	0.00
Tryptophane.....	Present.	0.00
Ammonia.....	3.46	3.64
Total.....	74.87	85.27

\* Osborne, T. B., and Liddle, L. M., *Am. J. Physiol.*, 1910, xxvi, 304.

#### EXPERIMENTAL.

500 gm. of the kafirin, equivalent to 473 gm. of ash- and moisture-free protein were hydrolyzed in three separate portions of 100, 200, and 200 gm. each by heating with 1,500 cc. of hydrochloric acid (specific gravity 1.1) on a steam bath until nearly all of the substance had gone into solution, and then boiling for 40 hours in an oil bath.

Glutaminic acid was separated from each portion by concentrating the solutions to about two-thirds of the original volumes, saturating with hydrochloric acid gas at 5–10°C., and allowing them to stand in a refrigerator for 5 to 6 days. The crystalline product was filtered off by suction on asbestos, washed with cold alcohol which had been saturated with dry hydrochloric acid gas, dissolved in water, and most of the color removed by means of



animal charcoal. The solutions were then boiled with an excess of barium hydroxide until nearly all of the ammonia had been expelled. The barium hydroxide was removed quantitatively with sulfuric acid. From the resulting solutions there were obtained 23.30, 42.97, and 49.15 gm. respectively of practically pure glutaminic acid hydrochloride, equivalent to 92.50 gm. of the free acid, or 19.55 per cent of the kafirin. The glutaminic acid hydrochloride gave the following analytical results:

*Carbon and Hydrogen*.—0.3487 gm. substance gave 0.4167 gm.  $\text{CO}_2$  and 0.1769 gm.  $\text{H}_2\text{O}$ .

*Nitrogen*.—0.1825 gm. substance required 10.3 cc. 0.1 N  $\text{H}_2\text{SO}_4$ .

*Chlorine*.—0.2028 gm. substance gave 0.1580 gm.  $\text{AgCl}$ .

	C	H	N	Cl
Calculated for $\text{C}_5\text{H}_9\text{O}_4\text{N} \cdot \text{HCl}$ ,				
per cent.....	32.70	5.49	7.63	19.32
Found, per cent.....	32.59	5.68	7.70	19.26

The filtrates from the glutaminic acid hydrochloride were united and concentrated under diminished pressure to a thick sirup, which was taken up in absolute alcohol and again concentrated to remove water. The residual amino-acid hydrochlorides were then esterified according to the method of Phelps and Phelps (2) as follows: The sirup was dissolved in 50 cc. of concentrated alcoholic hydrochloric acid and 50 cc. of absolute alcohol. After adding 40 gm. of fused zinc chloride, the vapors of 3,500 cc. of absolute alcohol containing 105 cc. of alcoholic hydrochloric acid were passed, during 11 hours, through the solution which was kept at a temperature of 105–110°C. by heating in an oil bath. The esters were dissolved in about a liter of absolute alcohol. After standing over night about 32 gm. of ammonium chloride had separated, which was filtered off, washed with absolute alcohol, and the washings and the solution of esters were united, and made up to 2 liters with absolute alcohol. Chlorine was determined in two aliquot portions of 20 cc. each. The esters were liberated from their hydrochlorides by the addition of the calculated quantity of sodium dissolved in absolute alcohol so as to make about a 3 per cent solution of sodium ethylate. The sodium chloride was removed by decantation and centrifugation, and the alcohol by distillation at about 40°C. under diminished pressure. The alcohol was saved for further exami-

nation. The thick, sirupy residue of esters was dissolved in about 500 cc. of ice cold water, and the solution shaken out several times with ether. Finally solid potassium carbonate was added to the residue until a thick paste was obtained, and the mixture extracted with ether until the ether extracts were colorless, the temperature being kept below  $0^{\circ}\text{C}$ . The ether solutions of the esters were dried in the usual way by standing over anhydrous sodium sulfate.

The residue containing the potassium carbonate was freed from inorganic salts by dissolving in water and saturating with hydrochloric acid gas and concentrating on the steam bath and filtering off successive crops of salts. The salts removed were washed with absolute alcohol and the washings added to the mother liquor. This solution was then esterified as described above.

The ether was removed from the united esters by distillation at atmospheric pressure and 320 gm. of esters were obtained.

*Ether Distilled from the Esters.*—The ether which was distilled from the esters was acidified with alcoholic hydrochloric acid and allowed to stand for 3 to 4 weeks. There was no separation of glycine ester hydrochloride crystals. The ether was distilled off, the residue taken up in water, an excess of barium hydroxide added, and the solution boiled for 6 hours to hydrolyze the esters and remove ammonia. After removing the barium hydroxide and chlorine the solution was evaporated to dryness. Proline was extracted from the residual amino-acids with absolute alcohol. There was thus obtained 10.87 gm. of amino-acids insoluble in alcohol, which were added to the fraction similarly obtained from Fraction I of the distilled esters (see below).

The alcoholic proline extract was evaporated to dryness, taken up in water, and examined according to the method of Van Slyke for determining amino nitrogen. The total nitrogen was 0.1825 gm., the amino nitrogen, 0.06468 gm., making the difference, 0.1178 gm. which corresponds to 0.97 gm. of proline.

*Alcohol Distilled from the Esters at  $40^{\circ}\text{C}$ .*—The alcohol, which was distilled from the esters after their liberation from their hydrochlorides by means of sodium ethylate, was acidified with alcoholic hydrochloric acid and the solution concentrated to a thick sirup. 5 volumes of water were added and the esters saponified by boiling from 6 to 7 hours. The chlorine was removed

with silver sulfate and the solution evaporated to dryness. After removing the proline in the usual way, there remained 19 gm. of amino-acids insoluble in alcohol. The alcoholic extract of proline was added to that similarly obtained from Fraction I (see below).

The amino-acids insoluble in alcohol when subjected to fractional crystallization yielded 2.21 gm. of leucine and 16.03 gm. of alanine. Analysis showed the leucine to have the following composition:

*Carbon and Hydrogen.*—0.1021 gm. substance gave 0.2049 gm.  $\text{CO}_2$  and 0.0927 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_6\text{H}_{13}\text{NO}_2$ , per cent. ....	54.96	9.99
Found, per cent. ....	54.73	10.16

The alanine gave the following results when analyzed:

*Carbon and Hydrogen.*—0.2001 gm. substance gave 0.2969 gm.  $\text{CO}_2$  and 0.1422 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_3\text{H}_7\text{O}_2\text{N}$ , per cent. ....	40.41	7.92
Found, per cent. ....	40.47	7.95

The esters of the amino-acids after removal of the ether were distilled as follows, using liquid air to condense vapors:

Fraction.	Temperature of the vapors up to	Pressure.	Weight.
	$^{\circ}\text{C}$ .	mm.	gm.
I .....	92	15.0	113.5
II .....	115	0.46	38.0
Undistilled residue .....			128.0

*Fraction I.*—This fraction was saponified by boiling for about 8 hours with 10 volumes of water. The solution was evaporated to dryness and again taken down several times with absolute alcohol to remove water. To the amino-acids of this fraction were added those obtained from the ether distilled from the esters, together with 1 gm. of substance which had separated on long standing from the alcoholic extracts of proline. Proline was extracted by boiling with absolute alcohol. The amino-acids insoluble in

alcohol, which weighed 71 gm., were subjected to fractional crystallization. There were obtained 25 gm. of leucine which had the following composition:

*Carbon and Hydrogen.*—0.2088 gm. substance gave 0.4209 gm.  $\text{CO}_2$  and 0.1913 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_6\text{H}_{13}\text{NO}_2$ , per cent. ....	54.96	9.99
Found, per cent. ....	54.98	10.25

There was further obtained a fraction weighing 30 gm. consisting of a mixture of leucine and valine. These two acids were separated by means of their lead salts according to the method of Van Slyke and Levene (10). The lead leucine, which weighed 32 gm., equivalent to 17.82 gm. of leucine, gave the following results on analysis:

*Lead.*—0.2914 gm. substance gave 0.1889 gm.  $\text{PbSO}_4$ .

	Pb
Calculated for $\text{Pb}(\text{C}_6\text{H}_{13}\text{O}_2\text{N}_2)_2$ , per cent. ....	44.29
Found, per cent. ....	44.27

The filtrate from the lead leucine, after removal of the lead with hydrogen sulfide, was evaporated to dryness. The residue, after removal of acetic acid and ammonium acetate with the ether-alcohol mixture, weighed 11.69 gm. and was practically pure valine. Without having been recrystallized it gave the following results on analysis:

*Carbon and Hydrogen.*—0.2826 gm. substance gave 0.5284 gm.  $\text{CO}_2$  and 0.2406 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_5\text{H}_{11}\text{O}_2\text{N}$ , per cent. ....	51.28	9.47
Found, per cent. ....	51.00	9.53

The remainder of Fraction I, which weighed 28.36 gm., was separated into nine fractions. The ninth fraction which contained most of the remaining substance, contained 41.25 per cent of carbon indicating the presence of alanine contaminated with valine. This percentage of carbon also shows that there was little or no glycine present. After extensive fractional crystallization it was found impossible to effect a separation of the alanine and valine. The fractions were accordingly united and thoroughly mixed. Analysis showed it to contain 43.34 per cent of

carbon and 7.86 per cent of hydrogen, corresponding to 7.66 gm. of valine and 20.67 gm. of alanine in this mixture.

The united alcoholic extracts containing the proline, after standing for several weeks, were filtered from a small amount of substance which had separated out, and the filtrate was evaporated to dryness; the sirupy residue was dissolved in water and made up to 500 cc. The total nitrogen in this solution was 2.3090 gm., the amino nitrogen, 0.3273 gm., the difference, 1.9817 gm., corresponding to 16.29 gm. of proline. The proline contained in the remainder of the solution after having made the Van Slyke amino nitrogen determination, was isolated and weighed in the form of its copper salts. These contained 10.88 gm. of *l*-proline and 5.79 gm. of *dl*-proline, which after correcting for the losses involved in the Van Slyke analyses amounts to 19.19 gm. of proline.

The *l*-proline copper salt was converted into the free acid and identified in the form of the phenylhydantoin which crystallized in beautiful characteristic prisms, melting sharply at 143°C.

The air-dried *dl*-copper salt showed the following composition on analysis:

<i>Water</i> .—0.3449 gm. substance lost 0.0377 gm. H <sub>2</sub> O at 110°C.	
Calculated for C <sub>10</sub> H <sub>16</sub> O <sub>4</sub> N <sub>2</sub> Cu2H <sub>2</sub> O, per cent.....	H <sub>2</sub> O 10.99
Found, per cent.....	10.93
<i>Copper</i> .—0.2843 gm. substance dried at 110° gave 0.0770 gm. CuO.	
Calculated for C <sub>10</sub> H <sub>16</sub> O <sub>4</sub> N <sub>2</sub> Cu, per cent.....	Cu 21.81
Found, per cent.....	21.64

*Fraction II*.—The phenylalanine ester was extracted from this fraction with ether in the usual way and saponified by heating with concentrated hydrochloric acid. The resulting product was united with that obtained in a similar way from the undistilled residue.

The aqueous layer which remained after the extraction with ether was saponified by boiling with barium hydroxide, and 2.72 gm. of aspartic acid were isolated in the form of the barium salt. Analysis of the free aspartic acid obtained by decomposing the barium salt with sulfuric acid showed it to have the following composition:

*Carbon and Hydrogen.*—0.1644 gm. substance gave 0.2191 gm.  $\text{CO}_2$  and 0.0810 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_4\text{H}_7\text{O}_4\text{N}$ , per cent.....	36.09	5.26
Found, per cent.....	36.35	5.51

No glutaminic acid or copper aspartate could be obtained from the filtrate from the barium aspartate.

*Distillation Residue.*—The undistilled portion of the esters was mixed with 5 volumes of water and the mixture shaken in the usual way with an equal volume of ether. On standing there separated from the ether solution about 4 gm. of diketopiperazines which were filtered off and the ether was allowed to evaporate at room temperature. The residual esters were hydrolyzed in the usual way with concentrated hydrochloric acid. After removing a small amount of oil by extraction with ether, the dark, red solution was largely decolorized by treatment with animal charcoal. To this solution was added that similarly obtained from Fraction II.

On account of an admixture with the phenylalanine contained in this solution, of considerable leucine and a little tyrosine, chlorine was removed quantitatively from the mixture of amino-acid hydrochlorides and the whole subjected to fractional crystallization. The total amount of amino-acids thus obtained was equivalent to 10.65 gm. of phenylalanine, 19.51 gm. of leucine, and 2.38 gm. of tyrosine. The phenylalanine on analysis gave the following results:

*Carbon and Hydrogen.*—0.1743 gm. substance gave 0.4168 gm.  $\text{CO}_2$  and 0.1079 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_9\text{H}_{11}\text{O}_2\text{N}$ , per cent.....	65.45	6.66
Found, per cent.....	65.22	6.93

The leucine had the following composition:

*Carbon and Hydrogen.*—0.2536 gm. substance gave 0.5096 gm.  $\text{CO}_2$  and 0.2241 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_6\text{H}_{13}\text{NO}_2$ , per cent.....	54.96	9.99
Found, per cent.....	54.80	9.88

The aqueous layer remaining after the extraction with ether was saponified with barium hydroxide, and the latter removed

quantitatively with sulfuric acid. The solution was then concentrated and saturated with hydrochloric acid gas at a low temperature. On standing there separated 8.09 gm. of glutaminic acid hydrochloride. The free acid obtained by decomposing the hydrochloride with an equivalent amount of normal potassium hydroxide was analyzed with the following results:

*Carbon and Hydrogen*.—0.2291 gm. substance gave 0.3439 gm.  $\text{CO}_2$  and 0.1285 gm.  $\text{H}_2\text{O}$ .

	C	H
Calculated for $\text{C}_5\text{H}_9\text{O}_4\text{N}$ , per cent.....	40.81	6.12
Found, per cent.....	40.94	6.28

No copper aspartate could be obtained from the filtrate from the glutaminic acid hydrochloride.

*Aspartic Acid*.—47.27 gm. of ash- and moisture-free protein were hydrolyzed by boiling with 200 cc. of hydrochloric acid (specific gravity 1.1) for 50 hours and the aspartic acid was determined according to Foreman's method (9) by its separation, together with glutaminic acid, from the other products of hydrolysis, as the calcium salts. There were isolated 6.86 gm. of glutaminic acid in the form of the hydrochloride, and 2.22 gm. of copper aspartate equivalent to 1.07 gm. of the free acid. Analysis showed the copper aspartate to have the following composition:

*Copper*.—0.2061 gm. substance, air-dried, gave 0.0602 gm.  $\text{CuO}$ .

	Cu
Calculated for $\text{C}_4\text{H}_5\text{O}_4\text{NCu} \cdot 4\frac{1}{2}\text{H}_2\text{O}$ , per cent.....	23.07
Found, per cent.....	23.34

*Tyrosine*.—Four different determinations of tyrosine were made. A quantity equivalent to 47.27 gm. of ash- and moisture-free protein was hydrolyzed by boiling with 100 cc. of concentrated hydrochloric acid and 100 cc. of water for 48 hours. The solution was then concentrated under diminished pressure and again taken down two or three times with water to remove as much as possible of the hydrochloric acid. The residual sirup was dissolved in water, decolorized with charcoal, and made up to 1,000 cc. The amount of chlorine in the solution was determined and the calculated amount of normal potassium hydroxide added. By concentration of the solution there were obtained 1.17 gm. of

tyrosine equivalent to 2.48 per cent, which gave the following results when analyzed:

*Carbon and Hydrogen.*—0.1527 gm. substance gave 0.3350 gm. CO<sub>2</sub> and 0.0868 gm. H<sub>2</sub>O.

	C	H
Calculated for C <sub>9</sub> H <sub>11</sub> O <sub>3</sub> N, per cent.....	59.67	6.08
Found, per cent.....	59.83	6.36

A second determination made by hydrolyzing 47.30 gm. of the ash- and moisture-free protein by boiling for 35 hours with 150 gm. of sulfuric acid and 300 cc. of water, removing the sulfuric acid quantitatively with barium hydroxide, and concentrating to crystallization, gave 1.18 gm. of tyrosine equivalent to 2.45 per cent of the protein.

The third determination was made colorimetrically according to the method of Folin and Denis (7). A quantity of kafrin equivalent to 1 gm. of the ash- and moisture-free protein was hydrolyzed by boiling for 12 hours with 25 cc. of 20 per cent hydrochloric acid. The solution was decolorized with charcoal and made up to 100 cc. The tyrosine in the solution determined colorimetrically as described by Folin and Denis, was equivalent to 5.49 per cent of the protein.

A fourth determination of tyrosine was made as follows: A quantity of kafrin equivalent to 46.81 gm. of ash- and moisture-free protein was hydrolyzed for a period of only 12 hours by boiling with 200 cc. of 20 per cent hydrochloric acid. After removing as much as possible of the free hydrochloric acid by concentrating under diminished pressure, the residual hydrochloric acid was neutralized by adding the calculated quantity of normal potassium hydroxide. It was then possible to crystallize out 1.83 gm. of tyrosine which are equivalent to 3.91 per cent of the kafrin. This is 1.43 per cent higher than the best yield obtained by direct isolation in the experiments described above in which the protein was hydrolyzed for 48 hours.

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# THE QUANTITATIVE DETERMINATION OF PHOSPHORUS BY THE NEPHELOMETRIC METHOD.\*

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(Received for publication, September 19, 1918.)

The nephelometric method of phosphorus determination proposed by Pouget and Chouchak<sup>1</sup> has since been developed and used by Kober and Egerer,<sup>2</sup> Greenwald,<sup>3</sup> and Bloor.<sup>4</sup> In determinations made by this method, the phosphate is precipitated from its solutions by what I shall call the "strychnine molybdate reagent." This is prepared by treating a solution of sodium molybdate in hydrochloric acid and water with strychnine sulfate. The method of preparing the reagent and the special procedure by which the phosphate is precipitated will be described more fully later. The determinations which form the basis for this article were made with the Duboscq colorimeter converted to a nephelometer, as described by Bloor.<sup>5</sup>

For convenience of discussion the density of a phosphate suspension may be defined as a quantity proportional to the amount of phosphate contained in the solution from which it was precipitated; and its nephelometric value, as the amount of light which a given volume of it will reflect to the eye of the observer under the conditions to which it is subjected in the nephelometer. It is obvious that there will be no proportionality between the densities and the nephelometric values of phosphate suspensions

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<sup>1</sup> Pouget, I., and Chouchak, D., *Bull. Soc. chim. France*, 1909, v, 104; 1911, ix, 649.

<sup>2</sup> Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

<sup>3</sup> Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.

<sup>4</sup> Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 133, 145; 1916, xxiv, 452.

<sup>5</sup> Bloor, *J. Biol. Chem.*, 1915, xxii, 145.

except under certain very special conditions. For the same quantity of precipitated phosphate may be divided into a few large particles or into more numerous smaller ones; the nephelometric value will obviously be different in the two cases, though the density may be the same. For this reason the investigators who have made use of the nephelometric method have laid great emphasis on the necessity of precipitating the standard and test suspensions as nearly as possible at the same time and under the same conditions.

When this is done, the nephelometric values of different phosphate suspensions are roughly proportional to their densities. By this I mean that if two suspensions of different densities are compared in the nephelometer under proper precautions, it will be found that the lengths of column which must be exposed to the light in order to make the illumination equal in the two semicircles are roughly inversely proportional to the densities. If, for example, the two suspensions have densities of 9 and 10 respectively, and if a column of the former 30 mm. long is exposed to the light, it will be found that approximately 27 mm. of a similar column of the latter must be exposed in order to make the illumination equal in the two semicircles.

But the proportionality between difference in density and difference in nephelometric value is not exact; and it is a very important practical question whether the correction which must be made depends only on fixed factors, such as the constructional peculiarities of the nephelometer and the degree of difference between the two suspensions to be compared, or whether it depends also on variable factors, such as the temperature at which the two suspensions are made and the character of the reagent used in precipitating them.

Kober and Egerer and Bloor imply in their discussions of the method that they think the former alternative is the case. Thus Kober and Egerer give mathematical formulas to be used in calculating the density of suspensions from the readings given by them in the nephelometer;<sup>6</sup> and Bloor says:<sup>7</sup>

<sup>6</sup> Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2378, 2379.

<sup>7</sup> Bloor, *J. Biol. Chem.*, 1915, xxii, 143.

"Since the readings obtained from different solutions are not exactly proportional to the amount of phosphate present and since the differences between the observed and theoretical reading increase as the differences in phosphate content increase it is necessary to calibrate the instrument for different strengths of test solution and for different standards, although where the test solution does not differ from the standard by more than 25 per cent the corrections fall within the limit of error of the determination and no correction can be made."

My own experience leads me to dissent from the view implied in the quotations given above, though I still think that the nephelometric method is of great value. The importance of a method by which 0.03 mg. of phosphorus can be quantitatively determined with even approximate accuracy is so obvious, that I feel justified in reporting my experience in some detail.

I have had occasion to make several hundred nephelometric determinations of the phosphorus content of known solutions. Of these, perhaps 50 per cent fell within 5 per cent of what was known to be the correct value; the others were off, sometimes as much as 30 per cent. There were two possible ways of accounting for the incorrect results. One was to suppose that they were due to accident—unconscious mistakes, contaminations by volatile materials in the air of the laboratory, by dust, etc. Another was to suppose that they were due to influences which are controllable, but which had not yet been controlled; such, for instance, as the temperature prevailing during various parts of the procedure and the age of the reagents.

My subsequent experience shows that temperature and the character of the reagent as determined by its age and by other factors are matters of the greatest importance. Very large deviations from what may be called the theoretical readings may be caused by the character of the reagent and by the amount of hydrochloric acid present at the time the phosphate suspensions are made, and it will therefore be necessary to consider these matters in some detail.

My strychnine molybdate reagent has been made up according to the description given by Kober and Egerer.<sup>8</sup> Specially pre-

<sup>8</sup> Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii. 2374-2376.

pared sodium molybdate,<sup>9</sup> water, and hydrochloric acid are mixed together in certain proportions, and to this solution a small amount of a saturated strychnine sulfate solution is added. The filtrate obtained from the resulting precipitation is used as the reagent. My determinations have generally been made according to the procedure given by Bloor.<sup>10</sup> 25 cc. of water are placed in each of two 50 cc. flasks, and 5 cc. of "1:1 HCl" and 5 cc. of the reagent are added in each case. 10 cc. of the standard phosphate solution are then run into one of the flasks, and 10 cc. of the test solution, into the other; the resulting suspensions are made up to volume with water, and subsequently read against each other in the nephelometer.

Kober and Egerer assert that the strychnine molybdate reagent as made up by them with hydrochloric acid is stable and gives quantitative and constant results.<sup>11</sup> This statement is not in entire accord with my experience. I have found that the stability and other important qualities of the reagent depend, to a surprising extent, on the strength of hydrochloric acid used in preparing it, and this concentration should be known with great exactness.

Both Kober and Egerer and Bloor use the phrase "1:1 hydrochloric acid" in the descriptions of their procedures, by which they mean acid made by adding together about equal parts by volume of the "concentrated hydrochloric acid" which is supplied

<sup>9</sup> The sodium molybdate was in most cases prepared as described by Kober and Egerer from Merck's molybdic acid "highest purity." The product obtained from this preparation is pure white crystalline powder, 15 gm. of which readily dissolve in 30 cc. of water, leaving no residue. Reagents made from such sodium molybdate are colorless when made, and remain so indefinitely. Lately, I have been unable to obtain Merck's molybdic acid, and have used a Baker and Adamson preparation, which is stated on its bottle to contain 12 to 15 per cent of ammonium nitrate. Reagents made from this preparation can be used fairly satisfactorily, though they are somewhat yellow when made and turn a deeper yellow on standing, probably on account of the nitric acid present.

The great majority of the nephelometric determinations and all the experiments reported in this article were carried out with reagents made from the Merck preparations, and the conclusions formed in this article are all drawn from experiments in which the Merck preparation was used.

<sup>10</sup> Bloor, *J. Biol. Chem.*, 1916, xxiv, 452.

<sup>11</sup> Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2374.

commercially, and water. In the subsequent discussion I shall give the concentration of solutions in terms of per cent by weight, unless otherwise indicated. By a "20 per cent solution of hydrochloric acid," for instance, a solution, which contains 20 gm. of HCl and 80 gm. of water, is indicated.

The chemically pure concentrated hydrochloric acid which we have obtained is advertised on its bottles to contain 37 per cent HCl. As a matter of fact, however, it has generally contained only about 35 per cent. Differences of this order in the concentration of hydrochloric acid used produce very marked differences in the character of the resulting strychnine molybdate reagent.

One gathers from the figures given by Kober and Egerer that their "1:1 hydrochloric acid" was of various strengths. In their article,<sup>12</sup> for instance, they direct that the 1:1 HCl be made by diluting 50 cc. of HCl with a specific gravity of 1.20 to 100 cc. with water. On the other hand, they state<sup>11</sup> that their 1:1 HCl had a specific gravity of 1.098. If it may be supposed that the specific gravities were taken at 15°, as is customary, the first solution would have a concentration of over 21 per cent, and the second, of 19.5 per cent. If the strychnine molybdate reagent is made up with 1:1 hydrochloric acid containing 21 per cent HCl and if the determination is carried out as Bloor directs,<sup>10</sup> no precipitate at all is obtained with such amounts of phosphate as are used by Kober and Egerer and by Bloor in their determinations.

To carry out the determinations as Bloor directs, therefore, it is necessary to use 1:1 hydrochloric acid weaker than 21 per cent. I have made up batches of reagent with various strengths of HCl ranging from about 16 to 21 per cent. The weaker the hydrochloric acid used, the less is the amount of the material precipitated from the solution of sodium molybdate in HCl and water by the addition of the strychnine sulfate solution. Reagents made up with weak HCl are unstable in the sense that a precipitate forms in them spontaneously on standing. In reagents made up with stronger 1:1 HCl more material is precipitated when the strychnine is added, and the spontaneous precipitate forms more slowly. But in all reagents which can be

<sup>12</sup> Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2375.

used in carrying out the Bloor procedure, so far as my experience goes, the spontaneous precipitate does form; it is only a question of its being formed more or less rapidly. Another change takes place gradually in all the reagents, which will be fully described below.

Reagents made up with weak hydrochloric acid tend to exaggerate the difference in nephelometric value as between phosphate suspensions with a given difference in density, whereas reagents made up with strong hydrochloric acid tend to minimize the nephelometric differences. As examples of the truth of this statement, the following experiments may be given, of which the results are shown in Table I.

Two reagents were made up in exactly the same way, except that 16.15 per cent HCl was used for one, and 19.10 per cent HCl for the other. Phosphate suspensions of different densities were then precipitated according to Bloor's procedure and read against each other, first one and then the other reagent being used in making the pairs of suspensions. The same pairs of phosphorus solutions were used in each of the three cases in making up the pairs of suspensions read against each other with the different reagents. The same sort of statement applies to all the other experiments reported in this article. In each case given amounts of the same pair of phosphorus solutions were precipitated first with one reagent and then with another, or first at one temperature and then at another temperature. The experiments, therefore, demonstrate in the strictest possible manner that the discrepancies between densities and nephelometric values were the result of the character of the reagent used or of the temperature at which the suspensions were made. They could not have been due to any accidents or mistakes in making up the phosphate solutions.

The results given in Table I bring out two interesting points. These points have been confirmed in other experiments which it has not been thought worth while to publish in detail. For convenience in discussing them, it may be said that by the theoretical nephelometric readings is understood readings which would indicate an exact proportionality between the densities of the suspensions and their nephelometric values, these terms being used in the senses indicated on pages 333 and 334.

Perhaps the most important point brought out in Table I is the fact that the differences in nephelometric value may be either greater or less than the differences in density. When the 0.000095 and 0.000114 per cent solutions are precipitated with the reagent containing weak HCl, the difference in nephelometric value is exaggerated somewhat beyond the theoretical; whereas, in the case of the 0.000133 and 0.000152 per cent solutions precipitated with the other reagent, the difference in nephelometric

TABLE I.

	P concentrations in solutions from which suspensions were made	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	<i>per cent</i>		<i>mm.</i>	
Reagent made up with strong HCl.	$\begin{cases} 0.000095 \\ 0.000114 \end{cases}$	0.83	$\begin{matrix} 20.0 \\ 25.2 \end{matrix}$	0.84
Reagent made up with weak HCl.	$\begin{cases} 0.000095 \\ 0.000114 \end{cases}$	0.83	$\begin{matrix} 30.0 \\ 23.9 \end{matrix}$	0.80
Reagent made up with strong HCl.	$\begin{cases} 0.000114 \\ 0.000133 \end{cases}$	0.86	$\begin{matrix} 30.0 \\ 26.9 \end{matrix}$	0.90
Reagent made up with weak HCl.	$\begin{cases} 0.000114 \\ 0.000133 \end{cases}$	0.86	$\begin{matrix} 30.0 \\ 24.6 \end{matrix}$	0.82
Reagent made up with strong HCl.	$\begin{cases} 0.000133 \\ 0.000152 \end{cases}$	0.87	$\begin{matrix} 30.0 \\ 28.9 \end{matrix}$	0.96
Reagent made up with weak HCl.	$\begin{cases} 0.000133 \\ 0.000152 \end{cases}$	0.87	$\begin{matrix} 30.0 \\ 26.4 \end{matrix}$	0.88

value is very markedly minimized. A formula totally different from that given by Kober and Egerer would be needed to cover the former of these two cases. It is true that the hydrochloric acid used was considerably weaker than that recommended by these authors; but, in the latter of the two cases, where the hydrochloric acid used approached closely to one of the strengths recommended by them, the difference in nephelometric value was considerably more minimized than it should have been according to their formula. It is to be noted that the readings



obtained with the reagent containing less HCl are, on the average, closer to the theoretical than the others. On the whole, I have found it better to make up my reagents with 1:1 hydrochloric acid which contains less than 19 per cent of HCl.

Table I also shows that, other things being equal, the difference in nephelometric value between a pair of suspensions is more greatly minimized the greater their absolute density. This point is very well brought out in the case of the three pairs of suspensions precipitated by the reagent containing the strong acid. Where the densities are both low, the nephelometric readings are very near the theoretical; the nephelometric differences are somewhat minimized in the intermediate pair of suspensions; and very markedly so in the densest pair.

The situation illustrated in Table I may be summed up by saying that if the densities of phosphate suspensions are to be calculated from their nephelometric values by means of mathematical formulas, a different formula will be required for every different reagent used and for every different density of standard suspension.

If the  $\text{HCl-Na}_2\text{MoO}_4$  solution contains a still less concentration of HCl when the strychnine sulfate is added to it in making up the reagent, the differences in nephelometric value as between phosphate suspensions of moderately different densities may be much more exaggerated than in the examples which have just been given. Following a suggestion of Dr. Bloor, I made up a batch of reagent in a manner quite different from that which has been used as a matter of routine. In this case 15 gm. of  $\text{Na}_2\text{MoO}_4$  were dissolved in 30 cc. of  $\text{H}_2\text{O}$ , and there were then added 230 cc. of 19.10 per cent HCl, 650 cc. of  $\text{H}_2\text{O}$ , and 20 cc. of saturated strychnine sulfate solution. In making phosphate suspensions with this reagent 10 cc. of the dilute phosphate solution were added directly to 30 cc. of the reagent. The results are given in Table II.

The character of the reagent may also be markedly influenced by the temperature at which the  $\text{HCl-Na}_2\text{MoO}_4$  solution is precipitated by adding the strychnine sulfate solution. If this precipitation is carried out at a high temperature, the resulting reagent will exaggerate the differences in nephelometric value as between phosphate suspensions of different densities. Table III shows the high degree to which this exaggeration may attain.

In all the reagents with which I have had any experience, a precipitate tends to be formed gradually, and another change goes on. This latter change consists in a growing tendency to minimize the difference in nephelometric value as between phosphate suspensions of different densities. All my reagents have tended to reach a state as a result of which the phosphate suspensions precipitated by them show no differences in nephelo-

TABLE II.

	P concentrations in solutions from which suspensions were made.	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	<i>per cent</i>		<i>mm.</i>	
Reagent made up as described (p. 340).	$\begin{cases} 0.000095 \\ 0.000114 \end{cases}$	0.83	$\begin{matrix} 30.0 \\ 20.6 \end{matrix}$	0.69
Reagent made up by routine method.	$\begin{cases} 0.000095 \\ 0.000114 \end{cases}$	0.83	$\begin{matrix} 30.0 \\ 25.8 \end{matrix}$	0.86

TABLE III.

	P concentrations in solutions from which suspensions were made.	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	<i>per cent</i>		<i>mm.</i>	
Reagent precipitated at about 45°.	$\begin{cases} 0.000095 \\ 0.000114 \end{cases}$	0.83	$\begin{matrix} 30.0 \\ 18.0 \end{matrix}$	0.60
Reagent precipitated at room temperature (about 20°).	$\begin{cases} 0.000095 \\ 0.000114 \end{cases}$	0.83	$\begin{matrix} 30.0 \\ 25.8 \end{matrix}$	0.86

metric value, even though the solutions from which they were made contained quite different concentrations of phosphate. This change often goes on slowly; the discrepancies between the actual and the theoretical nephelometric readings may not reach a stage where they destroy the value of the determinations for several weeks.

My experience with the strychnine molybdate reagent may be summed up as follows: The greater the concentration of hydrochloric acid present when the strychnine sulfate is added, the

greater will be the amount of material precipitated at that time and the greater will be the stability of the reagent. Reagents made up with strong hydrochloric acid, however, tend to minimize the differences in nephelometric value as between phosphate solutions of different densities; none of the reagents that are serviceable when used in the procedure described by Bloor are entirely stable; they all tend to form a spontaneous precipitate and to reach a state in which they show no difference in nephelometric value as between phosphate suspensions of different densities.

The temperature at which the strychnine sulfate is added to the  $\text{Na}_2\text{MoO}_4\text{-HCl}$  solution in making up the reagent may have a considerable effect on its character.

There is still another factor which may cause a lack of proportionality between differences in the densities of suspensions and the differences in their nephelometric values; namely, the temperature at which the phosphate suspensions are precipitated. It is, of course, to be expected that if phosphate suspensions are made from equally concentrated phosphate solutions but at different temperatures, they will have different nephelometric values. But the point to be made here is somewhat different from this. In this case, two suspensions are made from solutions of different concentrations, both at a given temperature. Later two other suspensions are made from the same two solutions, but both at some other temperature. Under such circumstances it will be found that the nephelometric difference between the suspensions is minimized at the higher temperature. Table IV gives an example of the kind of results that are obtained in this way.

Though the results which have been reported contribute little to the theory of nephelometric values, they show that in making such determinations it is not sufficient to assure oneself that the standard and test solutions are precipitated at the same time and under the same conditions. Even though these requirements may be ideally realized, the relation between difference in density and difference in nephelometric value may vary widely under the influence of a number of factors, of which the most important is the character of the reagent used. To make anything like a complete study of these factors would require great care and much time, for they react on each other in such a way

as to make it very difficult to disentangle their individual influences. But by taking certain precautions, the nephelometric method can be used in a practical way, even with our present scanty knowledge regarding it.

It has been a practically invariable rule in my experience that, whatever the relation between difference in density and difference in nephelometric value, less dense solutions read against denser ones in the nephelometer always show a lower nephelometric value. One method, therefore, of escaping from the difficulties which have been described is to have on hand enough of the test solution for several determinations. It is well also to have a series of standard phosphate solutions of graded concentrations, the range of which should extend from the lower to the upper

TABLE IV.

	P concentrations in solutions from which suspensions were made.	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	<i>per cent</i>		<i>mm.</i>	
Suspensions precipitated at about 28°.	$\begin{cases} 0.00019 \\ 0.00027 \end{cases}$	0.70	$\begin{cases} 30.0 \\ 23.8 \end{cases}$	0.79
Suspensions precipitated at about 7°.	$\begin{cases} 0.00019 \\ 0.00027 \end{cases}$	0.70	$\begin{cases} 30.0 \\ 21.8 \end{cases}$	0.73

limit of the range within which the concentration of the test solution might lie. The test is first read against that standard which is guessed to be nearest to it; and, under ordinary circumstances, this preliminary reading should make it possible to pick out a standard for a second confirmatory reading, whose difference from the test will lie very close to the limits of accuracy attained in any ordinary chemical work.

It is necessary also, however, to test the reagent frequently by using it to read two moderately different standard solutions against each other. For if, as is commonly the case with old reagents and with reagents made up with strong hydrochloric acid, the differences in nephelometric value are strongly minimized, the experimenter may be deceived in the preliminary test into thinking that the standard and test solutions have nearly

equal concentrations, when this is not really the case at all. In my experience, it has been necessary to test the reagent in the manner described at least every other day.

It is obvious that when the determinations are carried out as described above, one is more likely to be deceived by a reagent that minimizes the nephelometric differences than by one that exaggerates them. But reagents which greatly exaggerate the nephelometric differences are likely to be very unstable. In my experience those reagents are likely to be most serviceable which give nephelometric readings about equal to what they should be theoretically. This condition is approximately attained for the Bloor procedure in summer (in the neighborhood of Washington) by using a 1:1 hydrochloric acid which contains 19 per cent HCl. When the temperature is lower, I have found it advantageous to use a 1:1 hydrochloric acid which contains from 17 to 18 per cent HCl.

My experience with the nephelometric method began with a period of some weeks which I spent in learning and practising it under Dr. Bloor's direction, and I take this opportunity of expressing my sincere thanks for his kindness and very essential help and advice.

## NOTE ON THE PREPARATION OF GULONIC LACTONE.

By F. B. LA FORGE.

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(Received for publication, August 25, 1918.)

In synthetic investigations in the sugar group, it is often necessary to prepare one sugar from another having a lower number of carbon atoms.

To attain this object, only one procedure has been found successful; namely, the cyanhydrin method of Kiliani and Fischer. In the practise of this synthesis, the sugar in question is brought into contact with about the theoretical amount of hydrocyanic acid in aqueous solution, a small amount of ammonium hydroxide is generally added as a catalytic agent, and the reaction is allowed to proceed for many hours or days. The resulting solution is then boiled with an excess of a barium hydroxide to effect the saponification of the two nitriles formed by the addition of the hydrocyanic acid to the sugar, and at the same time to expel the ammonia resulting from the saponification. The two epimeric acids resulting from this reaction are present in the solution as barium salts and it is then necessary to remove the base by means of sulfuric acid, after which the acids are isolated as lactones, salts, or other derivatives. The corresponding sugars are obtained by reduction of the lactones of the acids.

Although this method always produces good results, it is nevertheless possible in its application in certain instances at least, to introduce very marked improvements. Such an instance is for example in the preparation of gulonic lactone and, hence of gulose, from xylose.

The simplified method about to be described, makes possible the elimination of the operation of boiling with barium hydroxide; the saponification of the nitrile of gulonic acid being effected with a slight excess of sulfuric acid. The resulting ammonium sulfate

is allowed to remain in the solution and in no wise interferes with the crystallization of the lactone, which takes place on concentration of the reaction product.

It became evident from the experiment described below, as was shown by polarimetric readings, that in the preparation of gulonic lactone only about 6 hours are required for complete reaction between the xylose and hydrocyanic acid.

If the pure sugar was used for the synthesis, very little color developed during the reaction and the solutions were finally only light yellow. The yield of chemically pure, recrystallized gulonic lactone amounted to 55 per cent of the weight of the xylose employed.

There is no doubt that the method could be applied in the preparation of glucoheptonic lactone from glucose and, with proper modifications, in other instances. In case the lactones in question were soluble in alcohol, for example, the ammonia could be removed as the chloride by means of this solvent.

*Preparation of Gulonic Lactone.*—150 gm. of xylose were dissolved in 300 cc. of water and 30 gm. (slightly more than the equivalent amount) of hydrocyanic acid were added to the solution. Upon addition of a few drops of ammonium hydroxide, reaction began as was evident from the increase in temperature of the solution. A polariscopic reading taken after about 25 minutes showed a rotation in a 1 dm. tube of  $+2.5^\circ$ ; showing that even after that short time, the reaction was very far advanced. After four hours, during which time the temperature of the solution was not permitted to go above  $35^\circ$ , a second reading showed a rotation of  $+1.2^\circ$  and this value remained practically constant during the following 16 hours. At this stage 1 cc. of the solution, after boiling with a few drops of concentrated hydrochloric acid, gave a reduction of Fehling's solution, corresponding to 0.1200 gm. of  $\text{Cu}_2\text{O}$ , showing that 90 per cent of the reducing sugar had disappeared. For subsequent preparations, 6 hours were shown to be sufficient time for the addition of hydrocyanic acid to xylose.

Slightly over one equivalent (55 gm.) of sulfuric acid diluted with a small amount of water was added to the solution, which was then concentrated at once to a thick syrup. Crystallization began almost immediately, but the mass of syrup and crystals

was allowed to stand over night, after which the crystals were filtered off on silk with suction. The crude product was nearly pure white and the mother liquor only slightly colored. The presence of ammonium sulfate does not interfere with the isolation of the idonic acid which may be accomplished by the benzaldehyde method.<sup>1</sup>

The crude gulonic lactone may be recrystallized from water, in which case the mother liquor must be concentrated to recover the dissolved portion, or from 60 per cent alcohol. The yield of recrystallized material amounts to 55 to 60 per cent of the weight of the xylose used.

<sup>1</sup> Van Ekenstein, W. A., and Lobry de Bruyn, C. A., *Rec. trav. chim. Pays-Bas*, 1899, xviii, 305.





## THE DETERMINATION OF CARBON DIOXIDE IN CARBONATES.

By DONALD D. VAN SLYKE.

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(Received for publication, September 16 1918.)

The method described below was devised primarily for use in determination of the carbonate in bones which had been dried and pulverized, but not ashed. This material offered peculiar difficulties to analysis by the usual methods. The method adopted appears to be applicable to all carbonates, soluble or insoluble, in the absence of acids, such as hydrogen sulfide, that are highly volatile from water solution. The principle of rapid extraction of carbon dioxide from solution by means of reduced pressure, utilized in the author's method for determining bicarbonates in blood<sup>1</sup> has been combined with the familiar precipitation of carbonic acid by standard barium hydroxide solution and titration of the excess of hydroxide. Under the reduced pressure the transfer of carbon dioxide to the barium hydroxide solution can be completed in 3 minutes, and the fact that the entire process is carried out in a single closed vessel practically excludes error from loss of carbon dioxide.

The carbonate, either pulverized or in solution, is placed in the bottom of a tube 20 to 25 mm. in diameter which is placed in a 250 cc. suction flask (Fig. 1) containing an excess of 0.1 N barium hydroxide. When pulverized carbonate is weighed into the tube, care must be taken that the entire amount is placed at the bottom, as portions sticking to the walls may fail to be decomposed by the acid added later. Our practice has been to weigh the tube, slip into it a roll of glazed paper to protect the walls, pour in the substance, and reweigh the tube after withdrawing the paper. When the tube is in place (Fig. 1), the flask is evacuated to a pressure of 50 mm. or less, and the outlet is closed with the screw clamp. An excess of normal hydrochloric acid, usually about 5

<sup>1</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

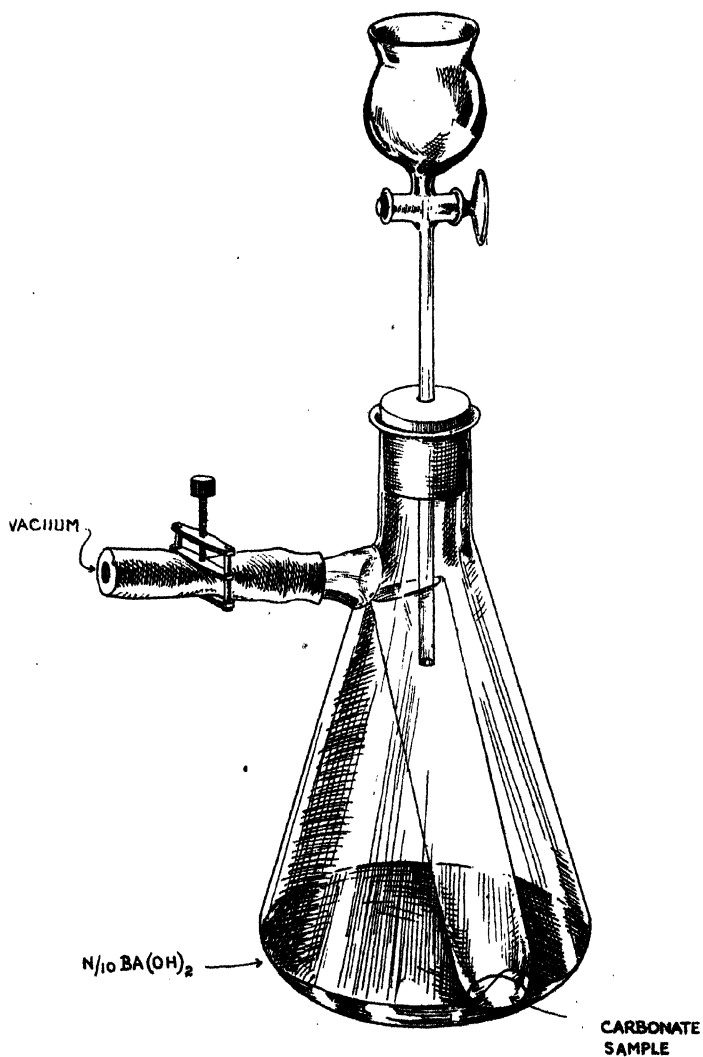


FIG. 1.

cc., is admitted slowly from the dropping funnel. Some of the solution is left above the stopper to assure its remaining air-tight. When the rapid evolution of carbon dioxide has ceased, the solutions are both agitated by a rotary motion for 3 minutes. In the analysis of sodium or calcium carbonate, this period has proved sufficient for complete transfer of carbon dioxide from the inner tube to the barium hydroxide solution. If the solution is not agitated, however, so that the surface of the barium hydroxide solution is continually renewed, the flask may stand for an hour without complete absorption of the carbon dioxide.

In the analysis of bones, which have been pulverized but not ashed, time must be allowed for the acid to penetrate into the particles, which are permeated with fat and protein. In this case it has been found necessary to allow at least 2 hours instead of 3 minutes for complete evolution and absorption of carbon dioxide. In routine analyses we have made a practice of allowing 5 hours. The solutions in the flask are stirred by rotation occasionally during this period, and for 3 minutes at the end of it.

After the reaction is completed the vacuum is released, and the barium carbonate is removed by filtration with a Gooch crucible, flask and crucible being rinsed with 3 portions of about 20 cc. each of water. The filtrate in the receiving suction flask is titrated against 0.1 N hydrochloric acid using phenolphthalein as indicator.

#### EXPERIMENTAL.

*Sodium Carbonate.*—A sodium carbonate solution was prepared containing 10 gm. of Merck's anhydrous  $\text{Na}_2\text{CO}_3$  per 100 cc. Titration with methyl orange indicated that the solution was of the calculated concentration. 1 cc. portions, measured to within 0.001 cc. with an Ostwald pipette, required 18.93 and 18.87 cc. of 0.1 N HCl, the calculated amount being 18.85.

*Calcium Carbonate.*—The calcium carbonate used was a Baker and Adamson's c. p. precipitated. To check its purity varying amounts were dissolved in excess 0.1 N hydrochloric acid, the carbon dioxide was expelled by boiling, and the excess acid titrated back with 0.1 N barium hydroxide, using alizarin sulfonate as indicator. The 0.1 N acid had been prepared by the method of Hulett and Bonner.<sup>2</sup>

<sup>2</sup> Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, **xxxi**, 390.

TABLE I.

CaCO <sub>3</sub>	0.1 N HCl	0.1 N Ba(OH) <sub>2</sub>	0.1 N HCl neutralised by CaCO <sub>3</sub> .		CaCO <sub>3</sub> in preparation used.
			Observed.	Calculated.	
gm.	cc.	cc.	cc.	cc.	per cent
0.4424	100.00	12.20	87.80	88.48	99.3
0.2060	50.00	9.29	40.71	41.20	98.8
0.2105	50.00	8.36	41.64	42.10	98.9
Average.....					99.1

In accordance with the above results, the weights of calcium carbonate taken in the following analyses were corrected by multiplication by the factor 0.991, and the weights given in Table II are so corrected.

TABLE II.

*Determination of Carbon Dioxide in Sodium Carbonate Solution and in Pulverized Calcium Carbonate.*

Na <sub>2</sub> CO <sub>3</sub> (in 10 per cent solution).	CaCO <sub>3</sub>	0.1 N Ba(OH) <sub>2</sub> in flask.	0.1 N HCl to titrate excess Ba(OH) <sub>2</sub> .	0.1 N Ba(OH) <sub>2</sub> precipitated by CO <sub>2</sub> .	CO <sub>2</sub> found.	CO <sub>2</sub> present.
gm.	gm.	cc.	cc.	cc.	gm.	gm.
0.100	—	29.00	10.20	18.80	0.0414	0.0415
0.100	—	29.00	10.15	18.85	0.0415	0.0415
0.100	—	29.00	10.20	18.80	0.0414	0.0415
—	0.0730	20.28	5.55	14.73	0.0324	0.0321
—	0.0909	20.28	2.00	18.25	0.0402	0.0400
—	0.1652	40.56	7.50	33.06	0.0727	0.0726
—	0.1709	40.56	6.20	34.36	0.0756	0.0752
—	0.1764	40.56	5.45	35.11	0.0773	0.0778

The accompanying paper by Dr. Goto gives the results obtained in bone analyses.

## MINERAL METABOLISM IN EXPERIMENTAL ACIDOSIS.

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(Received for publication, September 16, 1918.)

No detailed study of the mineral changes in the soft tissues and skeleton respectively, caused by either clinical acidosis or prolonged acid feeding, has been published so far as we have been able to ascertain. We have only the observation which Gerhardt and Schlesinger (1899) report to have been made by von Frerich, that the skeletons of diabetics dying in coma appear to be atrophied. Quantitative work on the subject appears to have been limited chiefly to a study of excretion.

Knowledge derived from direct analysis of the body itself has been limited to the blood. Walter (1877) showed that entrance of acids into the circulation causes an immediate reduction of the blood bicarbonate, and Palmer and Van Slyke (1917) have found that the bicarbonate concentrations of the body fluids in general parallel those of the blood plasma, so that depletion of bicarbonate in the blood plasma indicates a depletion of the bicarbonate stores throughout the body. When such a bicarbonate deficit continues, some depletion of the other alkali reserves might also be expected, and the literature contains a considerable amount of metabolic work which indicates that it occurs.

Ash analyses indicate that the main reserves of alkali aside from the bicarbonates are the calcium carbonate and the much greater amount of calcium phosphate in the bones, and the potassium and sodium phosphates of the tissues; *i.e.*, the alkali reserves aside from the carbonates are chiefly phosphates. It would therefore be expected that if the reserves of either the skeleton or soft tissues are drawn upon to a considerable extent, an increased excretion of phosphates would result, chiefly sodium and potassium phosphates if the source were the soft tissues, calcium

phosphate if it were the bones. Increased phosphate excretion in the urine has in fact been reported in diabetic ketonuria and in experimental hydrochloric acid feeding in man and in animals.

In diabetic ketonuria, however, it is uncertain whether the increased phosphoric acid excretion is due to loss of alkaline phosphates from the body, or merely to combustion of increased amounts of food and body proteins. Gerhardt and Schlesinger (1899) as evidence that there is an excretion of  $P_2O_5$  in excess of that accountable for by protein catabolism, report a  $\frac{P_2O_5}{N}$  ratio of  $\frac{18-20}{100}$  in the urines of two diabetics with heavy ketonuria.

The normal ratio under similar conditions was estimated at  $\frac{12.5}{100}$ . Feeding sodium bicarbonate to the diabetics reduced it to  $\frac{14-16}{100}$ . Rumpf (1898) and Mandel and Lusk (1904) report in a

fasting patient with severe diabetes  $\frac{P_2O_5}{N}$  ratios similar to those of Gerhardt and Schlesinger. Folin and Shaffer (1902), however, found similar ratios,  $\frac{18-20}{100}$ , common in normal adults.

Consequently, as stated above, the data at hand do not tell us whether or not the alkaline phosphates of the body are depleted in diabetic acidosis.

Increased excretion of calcium and magnesium in the urine has been reported in diabetic acidosis by Gerhardt and Schlesinger (1899) and by Tenbaum (1896). Lack of complete intake and outgo data, however, make it impossible to state whether the figures indicate a real loss of bone substance.

The metabolic experiments with acid feeding in the literature include observations more comprehensive than were made in the above cited studies on diabetics. Steenbock, Nelson, and Hart (1914) found that feeding hydrochloric acid to a calf increased the urinary phosphoric acid, but that even large doses showed no tendency to produce a negative phosphate balance. When the maximum dose of 400 cc. of 1 N HCl per day was given, a negative calcium balance was produced. The authors conclude that the acid attacked only the calcium carbonate, not the calcium phosphate of the bones.

Fitz, Alsberg, and Henderson (1907) observed in the urine of rabbits, which received dilute hydrochloric acid daily, a marked increase of phosphoric acid excretion, followed by a decrease, presumably when the readily available phosphates were exhausted. In some cases the excretion rose again before death.

Givens and Mendel (1917) examined the balance of nitrogen, calcium, magnesium, and phosphorus after the administration of base and acid in the dog. The phosphorus balance showed no significant effect and was always positive, though extremely variable quantitatively. The figures for both the urinary and the fecal phosphorus were so variable that neither could be used as an index of any influence exerted by sodium bicarbonate administration. Stehle (1917) states that the administration of hydrochloric acid by mouth to the dog causes an increased excretion of calcium and magnesium as well as of sodium and potassium.

Except for the results of Givens and Mendel (1917, 1918) with dogs, the above literature indicates that sufficient acid feeding produces in various animals an increased excretion of phosphate in the urine. The results of Fitz, Alsberg, and Henderson (1907), who followed the daily  $P_2O_5$  excretion of rabbits fed with HCl for weeks until death resulted, are particularly significant.

In our experiments the chief object has been to ascertain the effect of acid feeding on the mineral composition of the bones and muscles respectively; but we have followed the phosphate excretion in the urines of our rabbits in order to make certain that the animals were actually responding with increased phosphate excretion in the manner to be expected from the experiments of Fitz, Alsberg, and Henderson. The results were uniformly confirmatory.

There are however, two possible explanations of the increased phosphate excretion in the urine caused by acid feeding. The increased urinary phosphate may be drawn from the reserves of the body to neutralize the acid, or it may be due merely to increased absorption of calcium and magnesium phosphates of the food transformed in greater amount into soluble forms by the ingested acid in the alimentary tract. Würtz (1912) it is true failed to find that feeding hydrochloric acid to rabbits had any effect on their output of  $P_2O_5$  in either urine or feces. The



amounts of acid which he fed were, however, only about one-seventh as great as in the experiments of Fitz, Alsberg, and Henderson (1907), and for this reason his negative results are inconclusive. In order to ascertain whether the amounts of acid fed in our experiments influence the distribution of excreted phosphate between feces and urine, we have in some cases determined the excretion in both. The acid feeding showed no definite influence on the distribution. The increased phosphate excretion in the urine was not accompanied by a decrease in the feces, and consequently it appears that the urinary increase in our experiments indicates a genuine negative balance in phosphoric acid—a conclusion which is confirmed by the tissue analyses. These results lend added force to the conclusions of Fitz, Alsberg, and Henderson (1907), that their data indicated a utilization of body phosphates as alkaline reserve to neutralize invading acids and turn them into excretable salts.

#### EXPERIMENTAL.

##### *I. Effect of Acid Feeding on Phosphate Excretion in Urine and Feces.*

As experimental animals, rabbits were chosen in preference to dogs. The ammonia-forming power of dogs enables them to neutralize great amounts of acid without drawing upon the body reserves of mineral alkali. Rabbits can form ammonia much less rapidly (Walter, 1877), and can therefore be expected to neutralize unusual amounts of strong acid only by means of the reserves of alkali in the body fluids, tissues, and skeleton.

The rabbits were kept in metabolism cages and were offered daily 65 gm. of bread and 300 gm. of cabbage. They did not usually consume all that was given, as shown by Table VII. The acid was given in 0.25 N concentration by stomach tube, the amounts varying from 25 to 75 cc. daily. The conditions approximate those of Fitz, Alsberg, and Henderson (1907).

The urine was collected daily at 9 a.m. from Monday to Saturday, and kept in a refrigerator; the urine for the 5 week days was mixed and the total phosphoric acid estimated by titration with uranium nitrate.

Feces were collected daily at 3 p.m. before giving food, and the corresponding amounts of food and feces noted. Feces for 5 days, from Monday to Saturday, were mixed in one dish and ashed. The ash was dissolved by boiling with hydrochloric acid, and the solution was filtered into a 250 cc. volumetric flask and diluted to the mark. Phosphoric acid in 25 cc. portions was precipitated as molybdate and weighed as  $Mg_2P_2O_7$ .

*Results.*—The rabbits showed a poor resistance to hydrochloric acid, most of them dying within 1 or 2 weeks. Among the many

TABLE I.  
*Phosphorus Excretion in Urine of Normal Rabbits.*

Date of experiment.		PO <sub>4</sub> in urine per day.			
		Rabbit VIII (body wt. 1,500 gm.).	Rabbit X (body wt. 1,550 gm.).	Rabbit XV (body wt. 1,500 gm.).	Rabbit XVI (body wt. 1,400 gm.).
wk.	days	gm.	gm.	gm.	gm.
1st	1-6	0.039	0.057	0.037	0.032
2nd	8-13	0.035	0.044	0.066	0.032
3rd	15-20	0.043	0.074 Mixed with feces.	0.017	0.086 Mixed with feces.
4th	22-27	0.033	0.041	0.024	0.020
5th	30-34 (4 days)		.		0.063
Average ...		0.038	0.047 (1st, 2nd, 4th weeks.)	0.036	0.037 (1st, 2nd, 4th, 5th weeks).

rabbits, which were given 50 cc. of 0.25 N HCl daily from the beginning of the experiment, only Rabbit III lived more than 2 weeks. As the object of the experiment was to see the effect of continued acidosis, possibly similar to diabetic acidosis, upon the metabolic changes of the body tissues, it was intended to keep the rabbits at least 4 weeks. Therefore, 25 cc. doses of 0.25 N HCl were given to several of the rabbits. Of these, Rabbit V lived a long time and received increased doses of acid. Rabbits XI and XII received 30 cc. of 0.25 N HCl from the 2nd week.

TABLE II.  
*Excretion of PO<sub>4</sub> in Urine of Rabbits Fed with HCl.*

Date of experiment.		Acid-fed Rabbit III.			Acid-fed Rabbit IX.			Acid-fed Rabbit XX.			Acid-fed Rabbit XXI.		
		PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.	PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.	PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.	PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.
<i>wk.</i>	<i>days</i>	<i>gm.</i>	<i>gm.</i>		<i>gm.</i>	<i>gm.</i>		<i>gm.</i>	<i>gm.</i>		<i>gm.</i>	<i>gm.</i>	
1st	1-6	0.075	1,500	50 cc. 0.25 N HCl daily.	0.096	1,500	25 cc. 0.25 N HCl daily.	0.110	1,850	30 cc. 0.25 N HCl daily.	0.091	1,400	30 cc. 0.25 N HCl daily.
2nd	8-13	0.135	1,450	50 cc. 0.25 N HCl daily.	0.088	1,400	25 cc. 0.25 N HCl daily.	0.120	1,550	30 cc. 0.25 N HCl daily.			Died 7th day.
			1,310	Died 15th day.			Died 15th day.			Died 15th day.			

TABLE III.  
*Excretion of PO<sub>4</sub> in Urine of Rabbits Fed with HCl.*

Acid-fed Rabbit V.				Acid-fed Rabbit XII.				Acid-fed Rabbit XI.				Acid-fed Rabbit VII.			
Date of experiment.		PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.	PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.	PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.	Date of experiment.	PO <sub>4</sub> in urine per day.	Wt. of rabbit.	Remarks.	
wk.	days	gm.	gm.		gm.	gm.		gm.	gm.		wk.	days	gm.	gm.	
1st	1-6	0.122	1,450	25 cc. 0.25 N HCl daily.	0.037	1,650	25 cc. 0.25 N HCl daily.	0.078	1,300	25 cc. 0.25 N HCl daily.	1st	1-3	0.099	1,500	25 cc. 0.25 N HCl daily.
2nd	8-13	0.058	1,300	25 cc. 0.25 N HCl daily.	0.086	1,650	30 cc. 0.25 N HCl daily.	0.051	1,250	30 cc. 0.25 N HCl daily.	2nd	5-10	0.082	1,450	25 cc. 0.25 N HCl daily.
3rd	15-20	0.039	1,250	25 cc. 0.25 N HCl daily.	0.054	1,600	30 cc. 0.25 N HCl daily.	0.036	1,250	30 cc. 0.25 N HCl daily.	3rd	12-17	0.087	1,200	25 cc. 0.25 N HCl daily.
4th	22-27	0.080	1,250	35 cc. 0.25 N HCl daily.			Died 22nd day.	0.012	1,150	30 cc. 0.25 N HCl daily.					
5th	29-34	0.056	1,250	50 cc. 0.25 N HCl daily.				0.017	1,150	30 cc. 0.25 N HCl daily.					
6th	36-38	0.088	1,250	75 cc. 0.25 N HCl daily.						Died 35th day.					
				Died 38th day.											

Rabbits XX and XXI received 30 cc. of 0.25 N HCl from the beginning of the experiment, and lived less than 3 weeks, but were examined to ascertain the effect of the shorter period of acid feeding.

Four normal controls of almost similar weight showed an average daily  $\text{PO}_4$  excretion of 0.036 to 0.047 gm. (Table I).

TABLE IV.

*Phosphorus in Feces of Normal Rabbits.*

Date.		$\text{PO}_4$ per day.	
		Rabbit XV.	Rabbit XVI.
wk.	days	mg.	mg.
3rd	15-20	12.5	38.6
4th	22-27	40.7	87.5
5th	30-34		65.0

TABLE V.

*Phosphorus in Feces of Acid-Fed Rabbits.*

Date of experiment.		$\text{PO}_4$ per day.			
		Rabbit XI.	Rabbit XII.	Rabbit XX.	Rabbit XXI.
wk.	days	mg.	mg.	mg.	mg.
1st	1-6			39.6	56.9
2nd	8-13			49.5	
3rd	15-20	120	153		
4th	22-27	112			
5th	30-34	26			

Of the experimental rabbits, Nos. III, V, VII, IX, XI, XX, and XXI excreted more phosphate even in the 1st week than the controls (Tables II and III). Rabbit XII did not show the increased excretion in the 1st week, but did show an excessive excretion in the 2nd and 3rd weeks. Rabbit XI showed a normal excretion in the 3rd week, followed by diminished excretion in the 4th and 5th weeks. Rabbit V also showed a normal

TABLE VI.

*Diet of Normal Rabbits in Which Phosphoric Acid of Feces Was Determined.*

Date of experiment.		Rabbit XV.		Rabbit XVI.	
		Bread.	Cabbage.	Bread.	Cabbage.
wk.	days	gm.	gm.	gm.	gm.
3rd	15-16	65	300	65	300
	16-17	65	285	47	235
	17-18	50	187	52	200
	18-19	27	121	57	270
	19-20	37	130	52	202
4th	22-23	45	243	48	233
	23-24	58	223	58	300
	24-25	47	193	50	300
	25-26	61	220	54	222
	26-27	—	—	—	—
5th	30-31	—	—	58	300
	31-32	—	—	59	300
	32-33	—	—	43	172
	33-34	—	—	46	154

TABLE VII.

*Diet of Acid-Fed Rabbits in Which Phosphoric Acid of Feces Was Determined.*

Date of experiment.		Rabbit XI.		Rabbit XII.		Date of experiment.		Rabbit XX.		Rabbit XXI.	
		Bread.	Cabbage.	Bread.	Cabbage.			Bread.	Cabbage.	Bread.	Cabbage.
wk.	days	gm.	gm.	gm.	gm.	wk.	days	gm.	gm.	gm.	gm.
3rd	15-16	65	275	65	300	1st	1-2	—	300	—	300
	16-17	65	229	42	239		2-3	—	300	—	245
	17-18	53	166	56	200		3-4	40	200	—	157
	18-19	48	193	41	268		4-5	54	300	46	126
	19-20	49	148	38	171		5-6	38	300	31	98
4th	22-23	38	281	—	—	2nd	8-9	16	226	—	—
	23-24	47	204	—	—		9-10	45	293	—	—
	24-25	12	107	—	—		10-11	22	178	—	—
	25-26	19	168	—	—		11-12	17	133	—	—
	26-27	—	—	—	—		12-13	—	—	—	—
5th	30-31	44	171	—	—			—	—	—	—
	31-32	42	139	—	—			—	—	—	—
	32-33	31	143	—	—			—	—	—	—
	33-34	20	135	—	—			—	—	—	—

phosphate excretion in the 3rd week, but as he received gradually increased doses of acid after that period, the results are not comparable with those of Rabbit XI. When the amount of ingested hydrochloric acid rose, a corresponding increase of phosphoric acid excretion occurred.

The  $\text{PO}_4$  in the feces of the control rabbits was 12.5 to 87.5 mg. per day (Table IV), in the acid-fed rabbits 26 to 153 mg. (Table V). It is evident that the acid feeding caused no decrease in fecal phosphorus, the change being, if anything, in the opposite direction.

## *II. The Influence of Acid Feeding in the Rabbit on the Bicarbonate Content of the Plasma.*

Walter (1877), who made the first experiments in acid intoxication with dogs and rabbits, found great decrease in the carbon dioxide content of the blood. The results of later experimenters agree with Walter's. The effects of HCl administration in the dog upon the  $\text{CO}_2$  content of the plasma, estimated by the Van Slyke method, were recently reported by the writer (1917).

25 cc. of 0.25 N HCl were administered by stomach tube to several normal rabbits daily at 2 p.m. Blood was drawn by heart puncture before the acid feeding, and the  $\text{CO}_2$  content of the plasma estimated by the Van Slyke method (Table VIII). The rabbits died in about 1 week after the acid administration, since in their weakened condition they could not stand the heart puncture. Bicarbonate  $\text{CO}_2$  in the plasma of the controls was 44 to 64 per cent. In the 1st week of the acid administration (3rd day) several rabbits showed a lowered  $\text{CO}_2$  in the plasma. These rabbits did not live to the 2nd week. Rabbit XXVIII showed no acidosis in the 1st, but a marked acidosis in the 2nd week. Rabbit XXVII, which was not bled in the 2nd week but which received acid every day, also showed a marked acidosis in the 3rd week. Both rabbits died after heart puncture. Rabbit XI, used in one of the previous experiments, showed 34.9 per cent  $\text{CO}_2$  content in the plasma in the 4th week (April 30).

TABLE VIII.  
Carbon Dioxide Content of Plasma.

	Date.	Rabbit XXIV.	Rabbit XXV.	Rabbit XXVI.	Rabbit XXVII.	Rabbit XXVIII.	Rabbit XXIX.	Remarks.
		vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	
Normal condition.	May 21	44.0	44.0	44.0	57.2	60.0	53.4	
	" 24	54.4	66.0	63.9	51.5	57.3	63.0	
1st week of acid feeding.	May 29	31.2	45.6	36.9	51.1	63.8	43.6	From May 27.
		Died May 30.		Died May 31.			Died May 31.	25 cc. 0.25 N HCl given daily.
2nd week of acid feeding.	June 5		Died June 2.			33.6 Died June 5.		
3rd week of acid feeding.	June 12				31.2 Died June 12.			

### III. Mineral Changes in Muscles and Bones.

After the deaths of the rabbits which had been used for the phosphate excretion experiments reported above, the muscles and bones were separated as completely as possible. The total muscles (except the heart) were weighed, ground in a food chopper, weighed again, and kept in a refrigerator. From them phosphorus, potassium, and sodium were determined. The bones from the entire body, including the bone marrow, were weighed, dried on a water bath for 4 or 5 days, ground in a hand mill, weighed again, and kept in a bottle. In the bone powder, phosphoric acid, calcium, carbonic acid, and fat were estimated.

Age effects a change in the mineral constituents of the bones of rabbits (Weiske, 1872, Wildt, 1872), but the changes are mini-



mal in rabbits from 1 to 3 or 4 years of age. Adult animals from 1 to 2 years of about the same weight were selected for the present research.

*Methods of Analysis.*

*Phosphoric Acid in Muscle Ash.*—50 gm. of fresh muscle were weighed into a platinum dish and ashed, first over a flame and finally in a muffle furnace, using full red heat only after all organic matter was entirely turned to charcoal. To the residue 15 cc. of water and 3 to 5 cc. of concentrated hydrochloric acid were added, and the ash was dissolved with the aid of heat. The solution was washed into a 200 cc. flask, diluted to the mark, and filtered into an Erlenmeyer flask through a dry folded filter paper to remove particles of carbon. 50 cc. portions of this solution were taken for phosphate determination. The phosphate was precipitated as molybdate and weighed as  $Mg_2P_2O_7$ .

*Potassium and Sodium in Muscle.*—After ashing the muscle and dissolving the residue in the same manner, 5 cc. of 10 per cent  $BaCl_2$  were added and followed by 10 per cent  $Ca(OH)_2$  suspension till the solution was alkaline to litmus. The mixture was diluted to 150 cc. and filtered through a folded filter paper to remove phosphates and sulfates. To 100 cc. of the filtrate 20 cc. of saturated ammonium oxalate were added, followed by 10 per cent ammonium carbonate until no further precipitate was obtainable. The solution was diluted to 200 cc. and filtered through a folded filter paper to remove calcium and barium. 50 cc. of the filtrate were placed in a weighed silica dish with an excess of concentrated hydrochloric acid and evaporated to dryness on a water bath. The residual  $KCl$  and  $NaCl$  were ignited and weighed. The chlorides were dissolved in a small amount of water, 0.5 cc. of perchloric acid was added, and the solution evaporated to dryness. After addition of 3 or 4 cc. of water and five drops of perchloric acid, the solution was evaporated to dryness again. The residue was stirred up with 10 cc. of 97 per cent alcohol containing 0.2 per cent of perchloric acid, allowed to stand at least 20 minutes, and then transferred to a Gooch crucible, in which it was washed several times with small portions of the same alcoholic solution containing 0.2 per cent of perchloric acid. The  $KClO_4$  was dried at  $110^\circ C$ . and weighed.

*Phosphoric Acid Determination in Bones.*—10 gm. of dried bone powder were ashed in a platinum dish as above. The ash was transferred to a 200 or 300 cc. beaker, with 50 to 100 cc. of water. The latter was boiled and hydrochloric acid added until all solid matter except carbon was dissolved. The solution was filtered into a 250 cc. flask. The phosphate in 20 cc. was precipitated as molybdate and weighed as  $Mg_2P_2O_7$ .

*Calcium Determination in Bones.*—To 10 cc. of the bone ash solution, prepared as above, 5 cc. of 10 per cent sulfuric acid and 60 cc. of 95 per cent alcohol were added. The solution was let stand over night. The  $CaSO_4$  precipitate was filtered into an alundum crucible, washed with 70 per cent alcohol, and dried at  $100^\circ$ . The alundum crucible was then set inside a porcelain one, which was heated to a dull red for about 30 minutes.

*Carbonic Acid Determination in Bones.*—Van Slyke's new carbonate method was used (1918), as described in the preceding article. 1 gm. samples were used of the dried bone powder.

*Determination of Fat in Bone.*—1 gm. of dried bone powder was extracted in a Soxhlet extractor with 150 cc. of absolute ether for 24 hours. The ether was driven off on a water bath. 50 cc. of petroleum ether were added and let stand over night on the residue. The solution was filtered through fat-free cotton, the petroleum ether drawn off, and the residue weighed as fat.

## Results.

### I. Muscles.

1. *Weight of Muscles.*—The weight of the total muscles per kilo of body weight in four control rabbits was practically constant, varying only from 318 to 355 gm. (Table IX). In the experimental rabbits, the ratio of weight of muscles to body weight was calculated on the basis both of final body weight and of body weight before acid feeding was begun, since the animals lost weight during the period of acid feeding. The weight of muscles per kilo of initial body weight, except for Rabbits XII and XXI, was lower in the acid-fed rabbits than in any of the controls, indicating a loss of muscle tissue during acid feeding (Table X).

The weight of muscles per kilo of final body weight was lower than normal in Rabbits V, VII, IX, XI, and XX which had received a long continued acid feeding (13 to 38 days). The ratio in Rabbits XXI and XII was almost normal, in spite of the fact that they had received acid for 2 weeks or more. These

TABLE IX.

*Weight of Muscles in Normal Rabbits.*

Rabbit No.	Body wt.	Weight of muscles.		
		Before maceration.	After maceration.	
				Per kg. body wt.
	gm.	gm.	gm.	gm.
VIII	1,500	530.6	493.3	355
X	1,500	521.6	477.1	318
XV	1,500	544.0	523.8	350
XVI	1,400	476.8	449.0	321
Average.....				336

TABLE X.

*Weight of Muscles in Acid-Fed Rabbits.*

Rabbit No.	Duration of experiment.	Body weight.		Weight of muscles.		Weight of muscles.	
		Initial.	Final.	Before maceration.	After maceration.	Per kg. initial body wt.	Per kg. final body wt.
	days	gm.	gm.	gm.	gm.	gm.	gm.
III	15	1,500	1,310	430.5	415.0	277	320
V	38	1,450	1,250	335.2	319.5	220	255
VII	17	1,500	1,200	347.1	331.8	222	277
IX	13	1,500	1,400	442.1	424.0	283	303
XI	35	1,240	1,150	272.0	262.8	212	229
XII	22	1,650	1,600	579.0	561.0	340	350
XX	14	1,850	1,500	486.0	472.0	255	314
XXI	7	1,400	1,340	482.0	463.0	331	346

results show that such loss of weight as occurred fell more heavily on the muscles than on the body as a whole.

2. *Change in Phosphorus Content of Muscles.*—The control rabbits showed almost constant results, the percentage of  $\text{PO}_4$  in the fresh muscles being 0.759 to 0.883 gm., and the total  $\text{PO}_4$  per kilo of body weight being 2.6 to 2.8 gm. The percentage

of  $\text{PO}_4$  in the muscles of acid-fed rabbits was in some cases normal, but in four of the eight animals it was lower than the lowest normal. The ratio of total  $\text{PO}_4$  per kilo of initial body weight was remarkably lower than normal in all the rabbits which had received acid except Rabbit III. From the results it is evident that the total phosphorus in the muscles of rabbits is reduced by acid feeding, and that on the average, the reduction includes the percentage of phosphorus in the muscles as well as the amount in the total musculature.

3. *Changes in Sodium Content of the Muscles.*—The sodium content in the muscles of the normal animals showed an average of 0.0973 per cent, the extremes being 0.078 to 0.122. The total

TABLE XI.  
*Analysis of Muscle of Normal Rabbits.*

Rabbit No.	$\text{PO}_4$			Na			K		
	$\text{PO}_4$ in mus- cles.	Total $\text{PO}_4$ in all mus- cles.	Total $\text{PO}_4$ per kilo body wt.	Na in mus- cles.	Total Na in all mus- cles.	Total Na per kilo body wt.	K in mus- cles.	Total K in all mus- cles.	Total K per kilo body wt.
	per cent	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.
VIII	0.777	3.84	2.6	0.0988	0.487	0.33	0.301	1.49	0.99
X	0.883	4.21	2.8	0.122	0.582	0.39	0.252	1.20	0.80
XV	0.759	3.97	2.7	0.0903	0.473	0.32	0.32	1.68	1.12
XVI	0.796	3.58	2.6	0.078	0.351	0.25	0.385	1.73	1.24
Average...	0.804		2.64	0.0973		0.32	0.315		1.04

sodium per kilo of body weight was 0.25 to 0.39 gm., the average being 0.32 gm. for the four controls. A moderately high percentage of sodium is seen in the acid-fed rabbits, Nos. III, VII, and XXI. The ratio of the total sodium, both per kilo of initial body weight and per kilo of final body weight was lower than in the lowest control in Rabbits V, XI, and XX, which had received long courses of acid feeding.

4. *Changes in Potassium Content of the Muscles.*—In the normal rabbits the percentage of potassium in the muscles was 0.252 to 0.385, and the total potassium per kilo of body weight was 0.8 to 1.24 gm., the average being 1.04 gm. The percentage of potassium in the muscles of acid-fed rabbits, Nos. V, XII, and XXI, was lower than the lowest normal. The ratio of total potas-

TABLE XII.

*Analyses of Muscles of Acid-Fed Rabbits.*

Rabbit No.	Duration of experiment.	No. of days of acid feeding.	Total volume of 0.25 N HCl fed.	PO <sub>4</sub>				Na				K			
				PO <sub>4</sub> in muscles.	Total PO <sub>4</sub> in all muscles.	Total PO <sub>4</sub> in muscles.		Na in muscles.	Total Na in all muscles.	Total Na in muscles.		K in muscles.	Total K in all muscles.	Total K in muscles.	
						Per kg. initial body wt.	Per kg. final body wt.			Per kg. initial body wt.	Per kg. final body wt.			Per kg. initial body wt.	Per kg. final body wt.
	days		cc.	per cent	gm.	gm.	gm.	per cent	gm.	gm.	gm.	per cent	gm.	gm.	gm.
V	38	33	1,185	0.752	2.41	1.7	1.9	0.086	0.275	0.19	0.22	0.163	0.52	0.36	0.42
XI	35	29	810	0.827	2.17	1.8	1.9	0.084	0.221	0.18	0.19	0.251	0.66	0.58	0.59
III	15	12	600	0.933	3.87	2.6	2.95	0.132	0.584	0.37	0.42	0.288	1.195	0.80	0.91
XII	22	18	510	0.689	3.86	2.4	2.4	0.087	0.488	0.30	0.31	0.219	1.23	0.75	0.77
VII	15	15	375	0.595	1.99	1.3	1.7	0.141	0.467	0.31	0.39	0.268	0.896	0.59	0.74
XX	14	12	360	0.849	4.01	2.2	2.7	0.067	0.316	0.17	0.21	0.302	1.435	0.77	0.95
IX	13	12	300	0.733	3.11	2.1	2.2	0.0973	0.413	0.28	0.30	0.254	1.08	0.72	0.77
XXI	7	6	180	0.604	2.80	2.0	2.1	0.132	0.611	0.44	0.46	0.222	1.03	0.74	0.77

sium per kilo of initial body weight of all the acid-fed rabbits except No. III was lower than the lowest normal. The ratio of total potassium per kilo of final body weight in all the experimental animals, except Nos. III and XX, was lower than normal. It appears that potassium is considerably decreased in the muscles by experimental acidosis.

## II. Bones.

1. *Changes in Weight of Dried Skeleton.*—The weight of dried bone per kilo of body weight was 54 to 65 gm. in the normal rabbits and averaged 59 gm. (Table XIII). The weight of bone per kilo of initial body weight was lowered to 40 to 54 gm. in the acid-fed rabbits averaging 50 gm. (Table XIV). The

TABLE XIII.  
*Weight of Skeleton in Normal Rabbits.*

Rabbit No.	Wet.	After drying.	After crushing.	Per kg. body wt.
	gm.	gm.	gm.	gm.
VIII	140.5	86.2	80.8	54
X	158.9	94.4	83.0	55
XV	169.0	99.4	97.4	65
XVI	130.5	87.6	87.4	62
Average....				59

ratio of the weight of bone per kilo of final body weight was normal in the acid-fed rabbits, except in Rabbit V which had endured a long continued acid feeding. These results indicate that the dry weight of the skeleton decreases parallel with the weight of the total body during acid feeding. As shown below, about half the bone loss is attributable to decrease in the bone fat.

2. *Changes in Skeletal Phosphate.*—The  $\text{PO}_4$  percentage in the dried bones of normal rabbits was 20.8 to 23.3 and the total  $\text{PO}_4$  per kilo of body weight was 11.2 to 14.3 gm.

All of the acid-fed rabbits showed a percentage of  $\text{PO}_4$  in the bones rather higher than normal because of a decreased fat content, rather than an increase of phosphate, as will be indicated later. The ratio of the total  $\text{PO}_4$  per kilo of initial body weight

was lower than normal only in Rabbit V of the acid-fed rabbits. Rabbit V showed a smaller amount of all bone substances. Whether this was due to the exceptional amount of acid given or

TABLE XIV.  
*Weight of Skeleton in Acid-Fed Rabbits.*

Rabbit No.	Duration of experiment.	Weight of bone.				
		Wet.	After drying.	After crushing.	Per kg.	
	days	gm.	gm.	gm.	Initial body wt.	Final body wt.
III	15	109		71.8	48	55
V	38	119	61	58.5	40	47
VII	17	134		68.5	46	57
IX	13	148		81.1	54	58
XI	35	129	65.2	65.2	53	57
XII	22	166	90.4	88.4	54	55
XX	14	158	95	93.6	51	62
XXI	7	158	75.6	72.4	52	54

TABLE XV.  
*Analysis of Bone of Normal Rabbits.*

Rabbit No.	PO <sub>4</sub>			Ca			CO <sub>2</sub>			Fat.		
	PO <sub>4</sub> in dry bone.	PO <sub>4</sub> in total skeleton.	Total PO <sub>4</sub> per kg. body wt.	Ca in dry bone.	Ca in total skeleton.	Total Ca per kg. body wt.	CO <sub>2</sub> in dry bone.	CO <sub>2</sub> in total skeleton.	Total CO <sub>2</sub> per kg. body wt.	Fat in dry bone.	Fat in total skeleton.	Total fat per kg. body wt.
	per cent	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.
VIII	20.8	16.8	11.2	13.1	10.6	7.6	5.1	4.1	2.8	19.59	15.8	10.5
X	21.1	17.5	11.7	14.1	11.7	7.8	5.2	4.3	2.9	14.5	12.0	8.0
XV	21.7	21.0	14.0	14.7	14.3	9.5	4.8	4.7	3.1	18.21	17.7	11.8
XVI	23.3	20.3	14.3	15.2	13.3	9.5	4.9	4.3	3.1	16.22	14.1	10.1
Average.	21.7		12.8	14.3		8.6	5.0		2.95	17.13		10.1

to a chance individual variation from the normal, one cannot say. The bones of the other rabbits indicate no significant effect of the acid feeding on the phosphate content of the skeleton.

TABLE XVI.  
*Analyses of Bones of Acid-Fed Rabbits.*

Rabbit No.	Duration of experiment.	No. of days of acid feeding.	Total volume of 0.25 N HCl fed.	PO <sub>4</sub>			Ca			CO <sub>2</sub>			Fat.						
				PO <sub>4</sub> in dry bone.	PO <sub>4</sub> in total skeleton.	PO <sub>4</sub> per kg. initial body wt.	PO <sub>4</sub> per kg. final body wt.	Ca in dry bone.	Ca in total skeleton.	Ca per kg. initial body wt.	Ca per kg. final body wt.	CO <sub>2</sub> content of dry bone.	CO <sub>2</sub> in total skeleton.	CO <sub>2</sub> per kg. initial body wt.	CO <sub>2</sub> per kg. final body wt.	Fat in bone.	Fat in total skeleton.	Bone fat per kg. initial body wt.	Bone fat per kg. final body wt.
	days		cc.	per cent	gm.	gm.	gm.	per cent	gm.	gm.	gm.	per cent	gm.	gm.	gm.	per cent	gm.	gm.	
V	38		1,185	24.2	14.2	9.8	11.3	15.25	8.9	6.2	7.1	5.1	2.98	2.1	2.4	8.97	5.3	3.6	4.2
XI	35		810	25.7	16.7	13.5	14.6	17.1	11.2	9.0	9.7	4.7	3.06	2.5	2.7	3.53	2.3	1.9	2.0
III	15		600	24.9	17.9	11.9	13.6	16.8	12.1	8.0	9.2	5.1	3.66	2.4	2.8	9.88	7.1	4.7	5.4
XII	22		510	21.4	19.8	12.0	12.4	14.1	12.5	7.6	7.8	4.6	4.07	2.5	2.5	14.03	12.4	7.5	7.8
VII	17		375	27.2	18.6	12.4	15.5	16.85	11.6	7.7	9.7	5.0	3.42	2.3	2.9	7.02	4.8	3.2	4.0
XX	14		360	25.8	24.1	13.1	16.0	17.9	16.8	9.1	11.2	5.6	5.27	2.8	3.5	8.43	6.95	3.8	4.6
IX	13		300	25.1	20.3	13.6	14.5	16.25	13.2	8.8	9.4	5.2	4.20	2.8	3.0	6.94	5.6	3.6	4.0
XXI	7		180	22.8	16.5	11.8	12.3	13.8	10.0	7.1	7.5	4.7	3.26	2.3	2.4	9.99	7.2	5.2	5.4



3. *Changes in Skeletal Calcium.*—The percentage of calcium in the bones of the normal rabbits varied from 13.1 to 15.2, and the total calcium per kilo of body weight from 7.6 to 9.5 gm., the average being 8.6 gm. The acid-fed rabbits showed 13.8 to 17.9 per cent of calcium, and 6.2 to 9.1 gm., the average being 7.9 gm., of skeletal calcium per kilo of initial body weight. The last mentioned figures indicate that there was probably some loss of calcium during acid feeding.

4. *Changes in Skeletal Carbonate.*—The percentage of carbon dioxide in the bones of normal rabbits was 4.8 to 5.2; in the bones of acid-fed rabbits it was 4.7 to 5.6. The total  $\text{CO}_2$  per kilo of body weight was 2.8 to 3.1 gm. in the control rabbits; 2.1 to 2.8 in the acid-fed. The ratio of the total carbon dioxide per kilo of initial body weight of the acid-fed rabbits was lower than the lowest normal in six of the eight acid-fed rabbits. From these results it appears that the calcium carbonate of the bones, unlike the phosphate, is reduced by acid feeding. This conclusion is in agreement with that reached by Steenbock, Nelson, and Hart (1914) as the result of metabolism experiments on a calf. The fact that the *percentage* of  $\text{CO}_2$  is not reduced in bones of acid-fed rabbits is attributable to the loss of fat which makes the relative proportions of other constituents appear higher.

5. *Changes in Bone Fat.*—The percentage of fat in the bones of the normal animals was 14.5 to 19.6 averaging, 17.1, and the total fat per kilo of body weight was 8.0 to 11.8 gm. In the acid-fed rabbits, the fat percentage was 3.5 to 14.0 averaging 8.6 or half as high as in controls. The ratio of the total fat per kilo of initial body weight was correspondingly reduced. The reduction occurs even in the early stages of experimental acidosis. Because of this reduction in bone fat, the figures for the percentages of  $\text{PO}_4$  and Ca in the bones of acid-fed rabbits are rather higher than normal. The bone powder of the acid-fed rabbits was dryer and visibly less fatty than that of normal rabbits, and it was this appearance which led us to determine the relative fat content.

#### SUMMARY.

Daily administration of 25 to 75 cc. of 0.25 N hydrochloric acid to rabbits for 1 to 4 weeks had the following effects:

1. Blood plasma bicarbonate was reduced.
2. Phosphoric acid excretion was increased, the increase being in some cases followed by a fall. These observations confirm those of Fitz, Alsberg, and Henderson (1907).
3. Phosphate excretion in the feces was not markedly affected.
4. The muscles lost markedly in phosphorus and potassium and somewhat in sodium.
5. In the skeleton the chief effect was a great reduction in fat content, which fell from an average of 17.1 per cent of the dry weight to 8.6 per cent. Aside from the fat loss, the skeletons of acid-fed rabbits averaged about 10 per cent lighter in dry weight than those of the normals. There was no demonstrable loss of calcium phosphate. There was a definite loss on the average of about one-fifth of the carbon dioxide, indicating that the calcium carbonate is more readily sacrificed in acid intoxication than is the phosphate.

#### CONCLUSIONS.

The individual variations in muscle and bone are such, that in every constituent determined, the figures for some of the acid-fed rabbits overlap the normal range. Consequently the interpretation of results is not so sharp as could be desired, and it is regrettable that the time available has not permitted the extension of the experiments to a larger number of animals, so that the data could be put on a statistical basis.

The results nevertheless indicate with a fair degree of decisiveness that next to the bicarbonates of the body fluids, the first major reserves of alkali drawn upon in acid intoxication of rabbits are the alkali phosphates, particularly potassium phosphate, of the muscles, and the calcium carbonate of the bones. The bones do not lose calcium phosphate in appreciable amounts.

The above results are only suggestive in their relationship to clinical acidosis. Whether a loss of alkali phosphates from the tissues and calcium carbonate from the bones occurs in the latter remains to be ascertained by accurate determination of mineral balances.

The writer's thanks are due to Dr. Donald D. Van Slyke for his constant advice during the course of this work.

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# NOTES ON FOLIN'S DIRECT NESSLERIZATION METHOD FOR THE DETERMINATION OF NITROGEN.

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(Received for publication, September 17, 1918.)

Two difficulties were encountered in adopting Folin's direct Nesslerization methods<sup>1</sup> for nitrogen determinations in the urine and blood. The first was in making known solutions of pure ammonium sulfate check with the standard when run through as in the procedure for total urinary nitrogen. The second was that after precipitating the blood proteins with *m*-phosphoric acid the filtrate could not be boiled down in a large hard glass test-tube.

In order first to study the problem of the standard solution, two ammonium sulfate solutions were made up, one containing 1 mg. of nitrogen to 1 cc., and the other 1 mg. to 20 cc. All glassware was accurately calibrated, and Ostwald pipettes which delivered between marks were used for the 1 cc. measurements.

*Experiment 1.*—1 cc. of the more concentrated solution was digested with 1 cc. of the acid mixture in a large hard glass test-tube for 2 minutes after the sulfuric acid fumes had begun to come off, then diluted, and washed into a 200 cc. volumetric flask. 10 per cent sodium hydroxide was added in such amount as to make  $\frac{1}{8}$  of the volume necessary to neutralize 1 cc. of the acid mixture and 2 cc. for excess alkalinity, and the whole Nesslerized and diluted to volume. 20 cc. of the weaker solution were similarly treated after addition of the acid mixture but omitting the digestion. When compared in the colorimeter the color from the more concentrated solution of ammonium sulfate was more intense than that developed in the weaker solution without digestion. More color was brought out by digestion than could be accounted for by Nesslerization of the ammonium sulfate present so that the weaker solution could not serve as a standard for nitrogen determinations.

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<sup>1</sup> Folin, O., and Denis, W., Nitrogen determination by direct Nesslerization, *J. Biol. Chem.*, 1916, xxvi, 437.

*Experiment 2.*—In order to show whether the error involved in measuring such small amounts of ammonium sulfate or the possible loss in washing into the flask was considerable, 1 cc. of the more concentrated ammonium sulfate solution was put into each of two large hard glass test-tubes and the procedure concluded from this point as before, omitting the digestion. The readings checked exactly.

*Experiment 3.*—Experiment 2 was next repeated but one sample of the ammonium sulfate was digested for 2 minutes after the white acid fumes had begun to come off, before washing into a 200 cc. volumetric flask. The result was similar to that in Experiment 1. The color developed by the digested ammonium sulfate solution gave a reading of 9.3 when compared with the color from the undigested solution which was used as a standard and set at 10.

*Experiment 4.*—In order to show that the increase in intensity of the color developed after the digestion of the ammonium sulfate with the acid mixture was due to something in the acid itself, Experiment 3 was repeated using 1 cc. of distilled water in place of the ammonium sulfate solution. No color was developed by the undigested sample while there was a distinct brownish color after Nesslerization of the digested acid mixture.

On account of this impurity of the sulfuric acid now purchasable as C. P. we have found it necessary to make a correction for the color modification due to digestion or to digest our standard solution according to the following procedure.

1 cc. of a solution of purified ammonium sulfate containing 1 mg. of nitrogen to 1 cc. is measured with a calibrated Ostwald pipette into a large hard glass test-tube and digested for 2 minutes with a very low flame, a watch crystal being placed over the top of the tube when sulfuric acid fumes begin to come off. After cooling, distilled water is added, the whole washed into a flask, and set aside until the urine or blood filtrate has been similarly treated. The procedure is then completed as directed by Folin, and the solutions are compared in the colorimeter.

In the determination by direct Nesslerization of the non-protein nitrogen of the blood *m*-phosphoric acid is used as a precipitant. The resulting filtrate is water-clear and does not give the biuret reaction, but on account of bumping we were unable to boil it down in a large hard glass test-tube when the latter was held in the vertical position. If the tube is held just far enough from the horizontal to bring the surface of the liquid half way between the bottom and the mouth of the tube, the microburner flame adjusted so as to be not over  $\frac{3}{4}$  inch high with the tip of the

flame 1 cm. from the lowest portion of the tube, and moved away from the center line of the tube toward the edge, as in the accompanying figure, the fluid will boil without loss by bumping or foaming. Over 30 minutes will usually be required to boil the 10 cc. of filtrate down to 2 cc. but the tube need not be watched more than occasionally. When blackening begins the tube is turned upright and the remainder of the procedure carried out as in the case of urine. At this point, however, the precaution should be

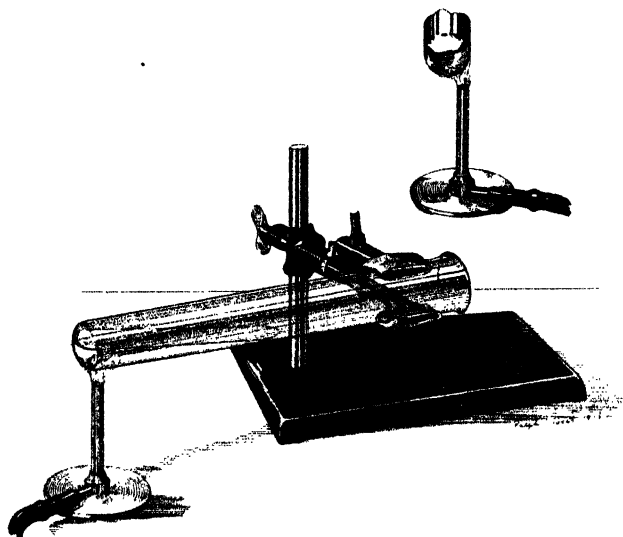


FIG. 1.

taken of placing the tip of the flame well over to one side, otherwise bumping and loss will occur. While keeping it in contact with the tube the flame should at the same time be lowered as far as is consistent with the continued digestion of the blackening filtrate, otherwise there will be too great a loss of sulfuric acid fumes and a consequent solidification of the material in the tube as digestion is completed. With the greatest care at the end of digestion solidification will sometimes occur and ruin the determination by making it difficult to wash the material quantitatively into a flask.

This can be prevented by raising the watch crystal slightly on one side and cautiously introducing distilled water with a capillary pipette and rubber nipple drop by drop down the side of the tube as soon as blackening disappears and the solution clears. Sputtering but no loss of material will occur. The remainder of the procedure is carried out as directed by Folin.

With these slight modifications we have found the method for determination of total urinary nitrogen and non-protein nitrogen of the blood by direct Nesslerization to be accurate and to effect a great saving of time and chemicals.

# ON THE DETERMINATION OF UROBILIN IN URINE.

## PRELIMINARY REPORT.

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(Received for publication. September 20, 1918.)

To the long list of methods of clinical investigation from which modern medical science has benefited may be added the determination of an increased quantity of urobilin in the urine of patients suffering from liver complaints.

Practically all modern investigators employ Schlesinger's method, which is based on the fact that even the smallest quantity of urobilin on the addition of certain zinc salts in an alcoholic solution produces a green fluorescence. As a matter of fact Jaffé had already called attention to this.

Schlesinger employed equal portions of urine and a 10 per cent suspension of zinc acetate in absolute alcohol. Hildebrandt points out that the reagent must be well shaken immediately before using, as a saturated solution of zinc acetate in absolute alcohol only contains 2 per cent and that to obtain the reaction it is sometimes necessary to work with large quantities of zinc acetate. Furthermore, he allows the mixture of urine and the reagent to stand for 12 to 24 hours before filtering in order that the urobilinogen present may be oxidized to urobilin. The same result is obtained instantly by the addition of minimum quantities of iodine.

In using Schlesinger's test in this way it was strikingly clear to us that the reagent, in the course of a few days, changed its appearance. While the *freshly* prepared suspension produced by finely pulverized zinc acetate and absolute alcohol was easily mixed by shaking, and on measuring gave practically uniform results with regard to the contents of zinc acetate in 10 cc., *this was not the case when the reagent was allowed to stand for a few days.* A freshly prepared suspension from which, after carefully



shaking, we measured 10 cc. into a porcelain bowl, evaporated the alcohol on a steam bath, and dried the remainder to a constant weight at 70°, gave as an average result of three tests 0.857 gm. of anhydrous zinc acetate, which corresponds to about 1.025 gm. of the hydrous zinc acetate used  $(\text{CH}_3\text{COO})_2\text{Zn} \cdot 2\text{H}_2\text{O}$ . A series of tests carried out in the same way after a lapse of 8 days gave, on the other hand, highly variable values from 0.27 to 0.82 gm. of zinc acetate.

On closer consideration the reason for this was obvious. The powdered zinc acetate had in the course of the 8 days changed into a coarse, crystalline, monoclinic zinc acetate which it was very difficult to shake up and which was very quickly precipitated.

To avoid the difficulty in the case of the prepared "Schlesinger's reagent" we decided to work, in all our experiments, with a weighed quantity of zinc acetate and a measured quantity of alcohol and urine.

Our method of procedure was as follows: Into one test-tube we weighed 1 gm. of zinc acetate and added to it 10 cc. of absolute alcohol; in another test-tube we measured 10 cc. of urine to which we added three drops of a 5 per cent alcoholic solution of iodine. We then carefully mixed the contents of the two test-tubes by repeated decantations until all the zinc acetate was dissolved. We finally filtered the precipitate and examined the filtrate for fluorescence in daylight falling from behind the observer through a test-tube 16 mm. wide.

On examining the urine of a number of patients suffering from different liver complaints, we obtained in this way very fine fluorescence results, but later on examining the urine of a number of normal persons and also of patients suffering from different surgical complaints, without any symptoms of liver involvement and with normal temperature, we found in the great majority of cases a very distinct fluorescence. It is, of course, well known that the urine of normal, healthy persons contains small quantities of urobilin. Gerhardt found 0.0074 to 0.0079 gm., Friederich Müller up to 20 mg., Sallet, 30 to 130 mg., and G. Hoppe-Seyler 80 to 140 mg. in the 24 hour urine. In other words the method proved too delicate in working with the exact quantities of reagent and urine.

Gregersen has, it is true, examined 800 patients apparently free from liver complaints and 50 normal persons, altogether over 5,000 urobilin tests, with Schlesinger's reagent and never found one positive reaction. A possible explanation of this striking disparity may lie in the fact that he, like all earlier investigators, used Schlesinger's reagent without considering the small quantity of zinc acetate he *might* in this way happen to work with.

In order to test the importance which the quantity of zinc acetate plays in the reaction we carried out the following experiment.

10 cc. of a slightly acid urine containing urobilin were measured into each of nine test-tubes. To each were added three drops of a solution of iodine. 10 cc. portions of absolute alcohol were measured into nine other test-tubes to which were added quantities of zinc acetate varying by 0.1 gm. from 0.9 down to 0.1 gm.

After careful mixing and filtering the experiments showed that the fluorescence was apparently unchanged in strength from 1 to 0.50 gm. Below that it decreased steadily until at 0.20 to 0.10 gm. it had to be considered as 0. Different urines, however, proved somewhat variable in this respect, but in the case of all of them it may be said *that the fluorescence was unaffected by varying the amount of zinc acetate from 0.5 gm. upwards, but that decreasing the quantity below 0.5 gm. diminishes the fluorescence obtained.*

We then examined the effect of alcohol concentration on the reaction.

As will be seen from Table I *the fluorescence decreases with the decreasing alcohol percentage.*

To complete the experiment the quantity of iodine was varied from half to threefold. This produced no visible change.

There seems to be some diversity of opinion with regard to the importance of the reaction of the urine. Hildebrandt states that highly concentrated acid urines do not give a reaction even when rich in urobilin, and, like most other investigators, he considers that a very weak alkaline reaction produces the best fluorescence results, while, for instance, Gregersen states that the reaction of the urine is of no importance whatsoever.

We decided, therefore, to try to throw some light on this point also by making the reaction on urobilin-containing urines to which were added N hydrochloric acid, N sulfuric acid, N acetic acid, or N solution of ammonia, in varying quantities but in such a way that the total volume remained constant.

TABLE I.

No.	Urine.	Iodine.	Zinc.	Water.	Alcohol.		Fluorescence.
					Absolute.		
	cc.	drops	gm.	cc.	cc.	per cent	
I	1	$\frac{1}{2}$	0.20	1	8	80	Distinct.
II	1	$\frac{1}{2}$	0.20	2	7	70	"
III	1	$\frac{1}{2}$	0.20	3	6	60	"
IV	1	$\frac{1}{2}$	0.20	4	5	50	"
V	1	$\frac{1}{2}$	0.20	5	4	40	Decreasing.
VI	1	$\frac{1}{2}$	0.20	6	3	30	"
VII	1	$\frac{1}{2}$	0.20	7	2	20	Very slight.
VIII	1	$\frac{1}{2}$	0.20	8	1	10	0
IX	1	$\frac{1}{2}$	0.20	9	0	0	0

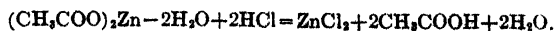
TABLE II.

No.	Urine.	Solution of iodine.	N HCl	Water.	Zinc acetate.	Absolute alcohol.	Filtrate.	
							Acidity, about	Fluorescence.
	cc.	drops	cc.	cc.	gm.	cc.		
I	5	2	0	5	0.50	10	Very slightly acid.	Strong.
II	5	2	1	4	0.50	10	0.05 N	"
III	5	2	2	3	0.50	10	0.1 N	Distinct.
IV	5	2	3	2	0.50	10	0.05 N	Slight.
V	5	2	4	1	0.50	10	0.2 N	Very slight.
VI	5	2	5	0	0.50	10	0.25 N	0

Table II shows the effects of the acids employed, Tables III. and IV those of the ammonia solution.

The tests were made on slightly acid urobilin-containing urines.

*The fluorescence of the filtrate decreases with the increasing quantity of the hydrochloric acid and disappears at the point where the zinc acetate is converted into zinc chloride, 0.50 gm. of zinc acetate requiring about 4.6 cc. of N HCl.*



In other words, in acid-reacting urines zinc chloride does not give any reaction, whereas a mixture of zinc acetate and zinc chloride does. This can be proved by the non-fluorescing filtrate (Test VI) fluorescing on the addition again of 0.50 gm. of zinc acetate and then filtering.

Sulfuric acid gave quite the same result, *whereas acetic acid added in equivalent quantities had no visible influence on the strength of the fluorescence.*

It was extremely interesting to observe the influence of ammonia on the reaction.

The urine employed had a slightly acid reaction and gave the urobilin reaction in a dilution of 1:20. The point where the filtrate shows the weakest fluorescence probably coincides with

TABLE III.

No.	Urine.	Solution of iodine.	N NH <sub>3</sub>	Water.	Zinc acetate.	Absolute alcohol.	Fluorescence.
		drops			gm.	cc.	
I	5	2	0	5	0.5	10	Strong.
II	5	2	1	4	0.5	10	Slight.
III	5	2	2	3	0.5	10	Very slight.
IV	5	2	3	2	0.5	10	" "
V	5	2	4	1	0.5	10	Slight.
VI	5	2	5	0	0.5	10	Strong.

the point where the solution holds the least quantity of zinc salt dissolved. On repeating the experiment with other urines the same conditions were discovered: *an increasing amount of ammonia gave first a decreasing and then again an increasing fluorescence*, but the minimum fluorescence of the different urines required the addition of a varying quantity of ammonia; in the same way the strengthening of the fluorescence required varying quantities of ammonia. In order to obtain this distinctly it was necessary in the case of urines rich in urobilin to use smaller quantities of urine, for instance 2 cc. instead of 5; and, in order to obtain the same total volume, an increased addition of water. Table IV shows a series of such experiments.

*In other words ammoniacal urines ought always to be acidified with acetic acid before making the test as it is impossible to know in advance whether the quantity of ammonia is such as to prevent*

entirely the reaction or to lead to an erroneous estimate of the quantity of urobilin. Gregersen's statement that the test can be made equally well on urines which have stood for several days may be wrong if the urine contains urea-decomposing bacteria. In the case of highly acid urines it is advisable to make alkaline with ammonia, then to acidify with acetic acid.

In order to discover whether the method could be employed to distinguish between normally and pathologically increased quantities of urobilin, we carried out a series of dilution tests. The aim of these was to ascertain how much it is necessary to dilute normal urine to avoid getting a positive reaction, and to find whether in liver complaints the quantity of urobilin is of such

TABLE IV.

No.	Urine.	Iodine solution.	N $\text{NH}_3$	Water.	Zinc acetate.	Absolute alcohol.	Fluorescence.
	cc.	drops	cc.	cc.	gm.	cc.	
I	2	1	1	7	0.5	10	Strong.
II	2	1	2	6	0.5	10	"
III	2	1	3	5	0.5	10	"
IV	2	1	4	4	0.5	10	Slight.
V	2	1	5	3	0.5	10	0
VI	2	1	6	2	0.5	10	Slight.
VII	2	1	7	1	0.5	10	Strong.
VIII	2	1	8	0	0.5	10	"

magnitude that the urine allows of greater dilution before the reaction fails to appear.

From our investigations into the importance of the alcohol percentage on the reaction we knew that in these tests we had to be careful that the alcohol percentage did not fall below about 40. We had likewise to make sure that the amount of zinc acetate was not too small. Fischler, who attempted such dilution experiments to form an estimate of the quantity of urobilin, made the fatal mistake of diluting the fluorescing filtrate with water, which prevents fluorescence even with very slight dilution. It appears, however, from the following experiment that by again increasing the alcohol percentage to the original quantity a distinct fluorescence reappears which is proportional in intensity to the amount of urine present. 10 cc. of a strongly fluorescing

filtrate are diluted with 10 to 15 cc. of water. The fluorescence disappears; then on the addition of 10 to 15 cc. of absolute alcohol a distinct fluorescence reappears. We cannot confirm Gregersen's observation that the fluorescence suddenly disappears at a certain point of dilution, if care is taken to keep the alcohol concentration at about 40 to 50 per cent.

In carrying out the dilution experiments in the manner stated below we found a quite uniformly decreasing fluorescence which in the case of urines rich in urobilin could still be observed with a dilution of 1:100. The difficulty at this point is to decide when the fluorescence may be described as non-observable, as even with a greater dilution we could determine a quite slight fluorescence, either by looking at the solution in a test-tube held horizontally, or by throwing a ray of light transversely across the glass with a convex lens.

In our experiment, however, we never availed ourselves of these accessories, and when we describe the fluorescence as 0, it means that the fluorescence was not observable in daylight falling from behind the observer through a test-tube 16 mm. in diameter.

We carried out these dilution tests in the following manner. 10 cc. of urine were measured drop by drop into test-tube A and to this were added three drops of a 5 per cent alcoholic solution of iodine. After shaking up, 1 cc. of this is measured into test-tube B.

I. To test-tube A, which now contains 9 cc. of urine, are added 9 cc. of absolute alcohol and 1 gm. of zinc acetate, and after complete solution of the zinc acetate the contents are filtered through a plain filter (9 cm. in diameter) into a test-tube of the width mentioned above (9 cc. of urine to 18 cc. of total volume; dilution  $\frac{1}{2}$ ).

II. If the filtrate fluoresces, to test-tube B are added 3 cc. of water, 1 cc. of a clear 20 per cent solution of zinc acetate in water, and 5 cc. of absolute alcohol. After mixing, it is filtered (1 cc. of urine in 10 cc. of total volume; dilution  $\frac{1}{10}$ ).

III. If the filtrate fluoresces, 5 cc. of urine are measured out drop by drop; to this are added 5 cc. of water and two drops of solution of iodine. From the mixture 1 cc. is measured into another test-tube and mixed with 3 cc. of water, 1 cc. of 20 per

cent zinc acetate solution, and 5 cc. of absolute alcohol, and then filtered. (0.5 cc. of urine in 10 cc. of total volume; dilution  $\frac{1}{20}$ .)

IV. If the filtrate fluoresces, 5 cc. of the urine are measured drop by drop into 15 cc. of water and two drops of iodine. From the mixture 1 cc. is measured, and the procedure is the same as in III (0.25 cc. of urine in 10 cc. of total volume; dilution  $\frac{1}{40}$ ).

In working in this way one is always certain of (1) *having a sufficient excess of zinc acetate*, (2) *sufficiently large and constant alcohol concentration*. Under these conditions it is possible to estimate the amount of urobilin in a urine. In a number of patients suffering from liver complaints the fluorescence could even be determined in dilutions of  $\frac{1}{40}$  to  $\frac{1}{20}$ , and in certain cases even with still greater dilutions. In a very few out of the 50 normal patients whose urine we have examined we have on one or two occasions seen the fluorescence in a dilution of  $\frac{1}{40}$ , and we should therefore be inclined to consider a fluorescence in a dilution of  $\frac{1}{20}$  as the lowest limit for a pathologically increased urobilinuria. But to decide this point more definitely a far greater number of investigations is necessary than we are able to carry out at present.

Perhaps it would not be out of place here to mention that the evaporation experiments conducted by Gregersen are based on a misunderstanding, as the urobilin is obviously a thermolabile substance.

We have made the following experiments. 250 cc. of slightly acid urobilin-containing urine (the fluorescence apparent in a dilution  $\frac{1}{40}$ ) were heated in a glass vessel in a water bath. The temperature of the urine reached its maximum of  $95^{\circ}$  in the course of 20 minutes. The urine was kept at this temperature for 1 hour. After restoring the water to its original weight from the loss it had undergone from evaporation and cooling, we conducted the urobilin test in the usual way. The fluorescence was now only visible in a dilution  $\frac{1}{20}$ . After remaining another hour on the water bath, restoring the water, and cooling as before, the fluorescence could only be seen in a dilution  $\frac{1}{2}$  and after a further hour's heating the fluorescence could not be observed at all.

## CONCLUSIONS.

The methods formerly employed for the quantitative determination of urobilin have either, as in the case of isolation and weighing, proved far too complicated for clinical use or, as in the case of spectroscopic methods of examination, not sufficiently delicate. By the dilution tests, here described, one, of course, does not obtain a definite figure for the quantity of urobilin present, but it is possible by a test, taking only a few minutes, to estimate sufficiently near for clinical use, how large quantities of urobilin a urine contains. We hope that further experiments with our method will be able to throw fresh light on several points related to it; for instance, on the importance of the concentration of the urine, the contents of pathological components, the variations in quantity of the substances usually present, the importance of the food, consumption of alcohol, and several other things which we hope later to be able to help in clearing up.





# STUDIES ON THE NUTRITION OF FISH.

## EXPERIMENTS ON BROOK TROUT.\*

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(Received for publication, August 27, 1918.)

Metabolic studies on aquatic animals have not been numerous. The inherent difficulties of collecting the excreta and measuring accurately the consumed food, both of which are soluble in the water, have doubtless made this subject unattractive to investigators.

In undertaking a nutritional study on the brook trout—a fish remarkably sensitive to slight changes in its environment—the first problem to settle was the practicability of keeping these animals in small aquaria. The trout thrive in rapid streams, and the problem would evidently be beyond solution if they could not be kept in good health in a limited quantity of water. Experience has shown that with sufficient aeration, trout can be kept in as little as 4 or 5 liters of water, which need not be changed but once every 48 hours, or even at longer intervals. Under such circumstances the trout will not only remain in good condition, but as the experiments here recorded show, will gain weight.

Large museum jars, about 12 inches in diameter, were used as aquaria, and these offered the fish considerable room for swimming, and could be kept very clean. The jar was closed by

\* This research was conducted with a special grant from the United States Bureau of Fisheries. The experiments were performed at the New York Aquarium and the Biochemical Laboratory of the College of Physicians and Surgeons, Columbia University. To Dr. C. H. Townsend, Director of the Aquarium and to Professor W. J. Gies of the Biochemical Laboratory, I desire to express my gratitude for the many courtesies received from them.

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a cover which could be clamped tightly to the bottom, and, by means of a flat rubber ring, the joint made air-tight. Two round holes drilled in the cover of the jar were fitted with rubber stoppers. Through one of these, the aeration tube was passed, while in the other a specially designed cup was inserted containing a measured quantity of standard acid. Compressed air was blown through the water, and was dissipated as a fine spray by the aeration tube. The air escaping from the jar bubbled through the standard acid contained in the cup. This precaution was taken in order to avoid any possible loss of ammonia from the water by the vigorous current of air.

The contents of the cup were emptied every 24 hours and titrated with 0.01 N sodium hydroxide. As no changes in the quantity of acid have been found in the course of many trials, this practice was discontinued as unessential to the accuracy of the experimentation.

The aquaria were submerged in a trough of galvanized iron through which a constant circulation of water of fairly uniform temperature was maintained. The temperature of the aquaria in which the trout sojourned was thus regulated. The water used in these experiments was exceptionally pure, coming directly from the excellent filters installed in the New York City Aquarium; no sediment was formed by this water even upon standing several weeks.

The trout which were experimented on were weighed at the beginning and close of each period. Different methods have been tested for obtaining the accurate weight of the fish, and finally the following method was adopted: The trout was picked up with a small fine net, the adhering water shaken off, and the trout allowed to drain for about half a minute. The fish was then transferred cautiously to an aluminum can partly filled with water, the weight of which was accurately determined. With practice it was possible to perform this operation without splashing a drop of water. The can was closed and again weighed with the trout, whose weight was thus gotten by difference. Though the method is not free from certain defects, it had two important advantages over every other method tried, in that, in the first place, no injury was done to the trout, and second, with the animal securely in the can, the weighing could be done lei-

surely. Of course it is assumed that the amount of moisture adhering to the animal has been the same at each weighing. Though this assumption is arbitrary, it has been found by weighing the animals several times in succession, that the extreme differences do not vary more than 0.5 per cent. The balance employed in all weighings was sensitive to a mg. with a load of 1.5 kilos.

Feeding the trout was unquestionably the most difficult, and yet the most essential step in the process of accurately measuring their metabolism. The effort, therefore, was made to teach the fish to take their food directly from forceps. Wherever this was feasible, the rest of the experimental procedure was quite simple. In a few instances I was actually successful in so training the trout that they would come to the edge of the aquaria and leaping out of the water, snap the food held with pincers. In this way the washing out of soluble constituents of the food by the water was entirely prevented.

The food was kept in small weighing bottles, and the amount consumed was determined by the difference in the weight before and after feeding. The method of feeding the trout *ad libitum* had the advantage also that at no time were there unconsumed particles of food left in the aquaria which might favor contamination of the water, and thus greatly affect the significance of the results.

Unfortunately this method of direct feeding could not always be utilized, as will be shown in a later section. The food was generally prepared in large quantity and stored in a frozen condition. The contents of every jar were carefully analyzed, and their composition was checked at least twice in the course of an experiment. Portions of this stock food—enough for several feedings—were put in weighing bottles and kept in the ice chest in the laboratory.

Usually 48 hours after feeding, the trout was removed to a jar with a fresh supply of water, and the old water containing the solid and dissolved excreta of the preceding period was filtered and prepared for analytical treatment.

Large aluminum tumblers, the bottoms of which were perforated with a number of fine holes, were used for this purpose, the tumblers serving as Gooch crucibles. The tumblers were provided with a thick pad of fine glass wool, dried in the oven, and

weighed. The contents of the aquaria were siphoned into the tumbler and filtered through the glass wool, with the aid of suction, into a large bottle. The glass wool was found very efficient in retaining even minute particles floating in the water, but the filtered water showed invariably a distinct turbidity. The tumbler with the solid excreta retained in the glass wool was again dried and weighed; thus the weight of dry feces was gotten by difference.

It may be mentioned that the sides and bottom of the aquaria were thoroughly cleaned with a rubber-tipped rod, and the wash water added to the filtrate. This was acidified with a few drops of sulfuric acid and evaporated to a small bulk. As a rule, the final volume was made up exactly to 1 liter.

In view of the large quantities of water that it was necessary to handle, the matter of evaporation presented certain technical difficulties. Originally it was attempted to carry out the evaporation at a low temperature (50°C.) with the aid of a rapid current of hot air. This method was very cumbersome, requiring much time. Blank experiments have shown that there was no particular advantage derived from the use of a low temperature. A large steam bath was therefore installed, accommodating a number of evaporating dishes (white enamel) of about 12 liters capacity each. The steam bath was set in the apparatus previously used for evaporating by means of the current of dry air, so that the two methods could be combined at will. Very large quantities of water could in this way be quickly condensed to a small bulk. The concentrated water was transferred to a volumetric flask, the evaporating dish thoroughly rinsed with fresh water, and the quantity brought up to a definite volume. This concentrated water was again filtered through asbestos to remove such particles as may have gone through the glass wool. The quantity was negligible, but the amount accumulated in the course of an experiment was analyzed and added to the feces. Aliquot portions of the perfectly clear water were used for analysis.

In choosing fine glass wool as a means of separating the solid excreta from the water, two important considerations were borne in mind; owing to the small quantity of feces available, it was very difficult to analyze it, and particularly to obtain a uniform sample. It was urgent, therefore, first to mix the feces

with some other material to increase its bulk without interfering with the analysis, and secondly, to make the grinding of the feces possible. The use of glass wool for this purpose suggested itself after a number of different things had been tried with little or no success. The glass wool proved particularly ideal as it served as a filtering medium, and could be ground to a thin powder, helping to reduce the feces to a state of extremely fine subdivision and uniform distribution.

These powders in which the glass represented many times the bulk of the feces, were kept in weighing bottles and were easily sampled for analysis. Nitrogen and fat determinations were made on weighed portions in the usual manner. It may also be mentioned that blank experiments were performed, and the analytical data furnished corrections for the dissolved excreta and feces which were found in experiments with trout.

#### *Experiments in Fasting.*

As a preliminary to the feeding experiments, a number were performed on fasting trout (Table I). It has been found that the alimentary tract frees itself of all excreta from previous feeding in 48 hours. Minute quantities were sometimes eliminated also during the next 24 hours, but this was invariably negligible; the fasting, therefore, was generally started 48 hours after the last meal. The first protracted fast was performed with a trout weighing 102.9 gm. (F-1). In the course of the 4 weeks of the experiment, no solid excreta were eliminated. The water in the aquarium remained remarkably clean for days, so that it could be changed at long intervals. The nitrogen eliminated in the water was determined for 7 day periods. After 28 days of fasting, the trout weighed 95.7 gm. or 7.2 gm. less than at the start. The nitrogen eliminated during the first week of fasting was 67.2 mg., but the quantity diminished from week to week, only 45.1 mg. being eliminated during the last week. The average elimination of nitrogen per day and per kilo of weight was 81 mg. In the course of 4 weeks 233 mg. of nitrogen were lost. It is evident, therefore, that about one-fifth of the body loss was at the expense of the protein.

The next two experiments were made with trout which had been kept previously in the stock tank and fed freely. Whe

TABLE I.  
*Experiments in Fasting.*

Experiment No.	Period of fasting.*	Average temperature of water. °C.	Weight.		Loss in weight. per cent	Total N excreted. gm.	N excretion per day. gm.	N excretion per day and per kg. gm.	Fat excretion (total). gm.	Fat excretion per day and per kg. gm.
			Initial.	Final.						
F-1	I. 25-II. 1	16.3	102.9	—	—	0.0672	0.0096	0.0941	—	—
	II. 1-II. 8		—	—	—	0.0629	0.0090	0.0900	—	—
	II. 8-II. 16		—	—	—	0.0583	0.0073	0.0745	—	—
	II. 16-II. 23		—	95.7	7.0	0.0451	0.0065	0.0670	—	—
F-2	VI. 4-VI. 11	17.2	52.2	49.7	4.6	0.0464	0.0072	0.1419	—	—
	VI. 11-VI. 18		49.7	49.1	1.3	0.0243	0.0035	0.0710	0.0043	0.0061
			—	—	5.9	0.0749	0.0054	0.1055	—	—
	VI. 5-VI. 12		82.4	81.0	1.7	0.0695	0.0099	0.1212	0.0082	0.0143
F-4	II. 10-II. 17	16.7	91.1	82.6	9.3	0.0965	0.0138	0.1559	—	—
	II. 17-II. 24		—	—	—	0.0458	0.0065	0.0793	—	—
			—	—	—	0.1423	0.0102	0.1171	—	—
	IV. 22-IV. 30		57.4	56.3	1.9	0.0415	0.0052	0.0916	—	—

\* The Roman numerals are used in the tables to designate the month of the year, the date being indicated by the arabic numerals.

transferred to the experimental aquaria, they vomited much undigested food, and for a few days continued to eliminate large amounts of feces. Trout F-2 in the 1st week of fasting lost 4.6 per cent of its weight, eliminating 141 mg. of nitrogen per day and kilo. This very high nitrogen elimination was followed by a decided drop in the next week, when it was only 71 mg., the loss in weight at the same time having been reduced to 1.3 per cent. During the entire 2 week period of fasting 5.9 per cent of the body weight was lost, and the daily nitrogen elimination per kilo of fish was 105 mg. The very high nitrogen elimination during the first few days of fasting which, in this case was preceded by abundant and unrestricted feeding, will be observed in several other experiments. This condition is met with also in the case of the higher vertebrates.

In Experiment F-3 it will likewise be observed that the nitrogen elimination reached a very high level of 132 mg. per day and kilo, but the fast was not continued further with this animal.

Experiment F-4 presents essentially the same picture. This trout was used in a long feeding experiment, and for weeks it was fed regularly and *ad libitum*. It was then subjected to a fast of 2 weeks duration, during which time it had lost 9.3 per cent of its weight. The nitrogen eliminated in the 1st and 2nd week shows that it was twice as large during the former, being 155 mg. of nitrogen per day and per kilo for the first 7 days, and only 79 mg. for the next 7 days. This large loss of nitrogen observed invariably upon changing from an abundant diet to fasting, especially in the case of Trout F-4 which for a month previous to the fast had been eliminating a fairly constant amount of nitrogen daily, brings up again the question of the circulating protein which, when the income of new food material is stopped, is the first to be consumed.

Experiment F-5 like the first of the series is interesting because this trout was not on an abundant diet previous to the fast. Its nitrogen elimination for 8 days was 91 mg. per day and kilo of body weight. We may regard, therefore, the elimination of 80 to 90 mg. per day and per kilo as the normal nitrogen catabolism of the brook trout.



*Feeding Raw Beef Heart.*

Raw beef heart cut in fine strips about 1 cm. long was fed directly from forceps. The results of an experiment which lasted over 2 months are recorded in Table II. During this time the excreta for every 48 hours were collected and analyzed separately. In the course of the 1st month, it will be seen, the trout gained 77.3 per cent in weight. It consumed 85.34 gm. of beef heart, and the excretion from day to day showed remarkable constancy. It will be noted also that the amount of nitrogen in the feces was almost invariably 10 per cent of the dry fecal matter, and this proportion coincides with that found in the feces of mammals on a pure meat diet. The dry feces constituted about 5 per cent (cf. Table VI) of the dry material ingested with the food. The utilization of the food—protein and fat—has been almost constant throughout this part of the experiment. Of the 2.8981 gm. of nitrogen fed, 0.9329 gm. was retained (cf. Table II, total N intake and total N eliminated). In the absence of experiments on the respiratory exchange, the retention of fat could not, of course, be worked out. Considering the amount lost with the feces, 96.1 per cent of the consumed protein was utilized, and 94.5 per cent of the fat. Not only has the utilization of the food through digestion been good, but as the "index of growth" (ratio between increase in weight and quantity of consumed food per kilo and per 24 hours) shows, it furnished a considerable proportion of its material to the building up of the body tissues.

The consumption of the trout per day and kilo of weight amounted to 37.5 gm. of food, while the increase in body weight per day and kilo was 17.4 gm. In other words, 46.5 per cent of the food material was added to the organism (see Table VI). The trout was then allowed to fast for a fortnight (F-4, Table I), in order to find out how this would affect the digestive power and general condition of the animal whose metabolic exchange has been carefully established by the previous study.

Upon resuming feeding, the trout gained 15.7 per cent in 2 weeks. During the first 4 days of realimentation, the small amount of nitrogen in the water (urine) is marked. During the first part of the experiment, the urinary nitrogen represented on the average 63.9 per cent of the total nitrogen consumption,

TABLE II  
Experiment 000 5. Raw Beef Hewt.

Average temperature	Period.	Initial or final weight.	Change in weight.	Amount of food.	N intake.	Fat intake.	Dry feces.	N in feces.	Fat in feces.	N in water (urine).	Total N eliminated.	N in feces in relation to N intake.	Fat in feces in relation to N intake.	N in water in relation to N intake.
°C.		gm.	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
16	I. 9-I. 13	51.4	—	6.07	0.2057	0.1718	0.0625	0.0153	0.0328	0.1089	0.3263	2.84	7.30	52.5
17.3	I. 13-I. 17	—	—	9.80	0.3324	0.2778	0.0840	0.0153	0.0328	0.2030	0.3263	2.84	7.30	61.1
17.9	I. 17-I. 21	—	—	9.10	0.3079	0.2572	0.0982	0.0269	0.0302	0.2085	0.4774	3.81	5.12	67.7
16.3	I. 21-I. 25	—	—	11.74	0.3083	0.3328	0.1568	0.0269	0.0302	0.2420	0.4774	3.81	5.12	60.8
15.8	I. 25-I. 27	—	—	5.57	0.1890	0.1578	0.0792	0.0068	0.0099	0.1211	0.1279	3.60	6.21	64.1
16.8	I. 27-I. 29	—	—	5.73	0.1995	0.1624	0.0832	0.0085	0.0094	0.1314	0.1399	4.27	5.79	65.9
15.1	I. 29-I. 31	—	—	5.94	0.2015	0.1683	0.0820	0.0084	0.0097	0.1338	0.1422	4.17	5.75	66.4
16.2	I. 31-II. 2	—	—	5.98	0.2029	0.1693	0.0835	0.0085	0.0102	0.1338	0.1423	4.19	6.02	65.0
15.3	II. 2-II. 4	—	—	5.48	0.1859	0.1552	0.0692	0.0075	0.0082	0.1208	0.1283	4.03	5.22	65.9
16.4	II. 4-II. 6	—	—	7.43	0.2521	0.2105	0.1060	0.0105	0.0093	0.1586	0.1691	4.17	4.42	62.9
16.0	II. 6-II. 8	—	—	7.13	0.2409	0.2021	0.0985	0.0104	0.0061	0.1638	0.1762	4.32	3.01	68.8
15.7	II. 8-II. 10	91.1	—	5.37	0.1820	0.1521	0.1050	0.0106	0.0139	0.1250	0.1356	5.82	9.13	68.7
16.4	I. 9-II. 10	39.7	+77.3	85.34	2.8981	2.4173	1.1120	0.1134	0.1397	1.8518	1.9652	3.91	5.78	63.9
16.7	II. 10-II. 24	82.6	-9.33	Fasting.										
17.5	II. 24-II. 26	82.6	—	4.48	0.1613	0.1219	0.0430	0.0048	0.0039	0.0641	0.0689	2.98	3.20	39.7
13.8	II. 26-II. 28	—	—	5.04	0.1814	0.1372	0.0680	0.0076	0.0061	0.1038	0.1114	4.19	4.45	57.2
15.0	II. 28-III. 2	—	—	4.06	0.1462	0.1105	0.0905	0.0082	0.0117	0.1551	0.1633	5.61	10.59	106.1
14.6	III. 2-III. 4	—	—	6.03	0.2038	0.1293	0.1210	0.0121	0.0169	0.1705	0.1826	5.94	13.07	83.2
15.4	III. 4-III. 6	—	—	4.40	0.1584	0.1198	0.0900	0.0107	0.0127	0.1318	0.1425	6.76	10.60	83.2
16.6	III. 6-III. 8	—	—	5.09	0.1832	0.1385	0.1160	0.0113	0.0109	0.1292	0.1405	6.17	7.89	70.5
15.2	III. 8-III. 11	95.5	—	6.54	0.2354	0.1789	0.1390	0.0113	0.0151	0.1732	0.1845	4.80	8.48	73.6
15.4	II. 24-III. 11	12.9	+15.7	35.64	1.2695	0.9352	0.6865	0.0660	0.0773	0.9277	0.9937	5.20	8.27	73.08

but during the first and second 48 hours following the fast, it was 39.7 and 57.2 per cent respectively. There was thus undoubtedly a retention of nitrogen. As the elimination for the next 48 hours shows, this retention was only temporary.

During this third 2 day period, the urinary nitrogen represented 106.1 per cent of the consumed nitrogen. In other words the nitrogen ingested since the fast was broken, was not excreted until 6 days later. The nitrogen elimination then gradually diminished, but even after 2 weeks, it was still 10 per cent higher than in the first part of this experiment.

An examination of the feces also revealed that the amount of dry excreta has become 50 per cent greater than during the preceding period, and furthermore that the feces became very fatty; in fact, from the 5th until the 10th day of resumed feeding, the per cent of fat in the feces was extremely high; this apparently was due to delayed elimination of undigested fat rather than to a progressive impoverishment of the digestive functions. In this respect there is almost a direct relationship to the excretion of urinary nitrogen. The utilization of nitrogen during these 2 weeks was 94.8 per cent, not essentially different from the degree of utilization in the prefasting period, but the fat utilization was only 91.7 per cent.

That the general condition of the trout was impaired by the fasting, is indicated best by the lowering of the "growth index" (see Table VI). During these 15 days the trout received 26.7 gm. of raw beef heart per day and per kilo of body weight, but it gained only 9.73 gm. in weight; in other words, the "growth index" was 36.5 per cent as compared to 46.5 per cent in the earlier part of the experiment.

The experiment recorded in Table III presents much similarity to the preceding one.

Six small fingerlings, weighing all together only 33.6 gm., were used. During the first part of the experiment, lasting 26 days, these small trout were fed thirteen times and their excreta were collected and separately analyzed four times during that period. A glance at Table III will show that the animals consumed 25.41 gm. of the raw beef heart, and gained 11.13 gm. in weight. The quantity of dry feces eliminated in that time was 3.52 per cent of the total dry ingested food, and contained 21 mg. of nitrogen.

TABLE III.  
*Experiment 000 6. Raw Bee! Heart.*

Average temperature.	Period.	Initial or final weight.	Change of weight.	Amount of food.	N intake.	Fat intake.	Dry feces.	N in feces.	Fat in feces.	N in water (urine).	Total N eliminated.	N in feces in relation to N intake.	Fat in feces in relation to N intake.	N in water in relation to N intake.
°C.		gm.	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
17.1	I. 19-I. 27	33.6	—	4.74	0.1609	0.1344	0.0368	0.0036	0.0056	0.1344	0.1380	2.24	4.17	83.5
15.9	I. 27-II. 2	—	—	5.09	0.1727	0.1443	0.0540	0.0047	0.0056	0.1245	0.1292	2.72	3.88	72.1
16.2	II. 2-II. 8	—	—	7.23	0.2455	0.2051	0.0566	0.0050	0.0069	0.1730	0.1780	2.04	3.36	70.5
16.1	II. 8-II. 14	44.7	—	8.35	0.2871	0.2404	0.0864	0.0079	0.0092	0.1962	0.2041	2.75	3.41	68.4
16.3	I. 19-II. 14	11.1	+33.4	25.41	0.8662	0.7239	0.2338	0.0212	0.0273	0.6281	0.6493	2.45	3.77	72.5
	II. 14-II. 18			Fasting.										
16.3	II. 18-II. 23	34.8	—	2.84	0.1022	0.0782	0.0375	0.0037	0.0067	0.1074	0.1111	3.62	8.57	105.1
17.5	II. 23-II. 27	—	—	4.00	0.1440	0.1104	0.0763	0.0074	0.0099	0.0982	0.1056	5.14	8.97	70.0
14.8	II. 27-III. 3	—	—	4.72	0.1699	0.1305	0.0650	0.0066	0.0097	0.1082	0.1148	3.89	7.43	63.7
15.2	III. 3-III. 7	—	—	5.86	0.2110	0.1618	0.0935	0.0099	0.0139	0.1335	0.1434	4.70	8.06	63.3
15.9	III. 7-III. 11	46.1	—	5.53	0.1990	0.1525	0.1025	0.0102	0.0119	0.1365	0.1467	5.13	7.80	68.6
15.9	II. 18-III. 11	11.3	+32.8	22.95	0.8261	0.6334	0.3748	0.0378	0.0521	0.5838	0.6216	4.58	8.23	70.7

The protein of the food, therefore, was utilized to the extent of 97.5 per cent, and the fat 96.2 per cent. The index of growth was 42.7 per cent showing that the fingerlings were growing at a favorable rate.

Owing to the accidental death of one of the animals, the experiment was renewed with the five remaining fingerlings, and continued for another 21 days. During this time the excreta were collected and analyzed five times. A short fast of 4 days intervened between the first and second parts of this experiment. The total weight of the five fingerlings was 34.8 gm., and they gained 11.3 gm. in the 21 days, or considering the average weight at the beginning and closing of the entire experiment, the fingerlings gained about 66 per cent in weight.

By reviewing the data pertaining to the second part of the experiment, certain differences will be observed as compared with the results obtained in the first part, which to a certain extent substantiate the experience gained in Experiment 000.5. In the first place, the ratio of dry feces to the dry matter of food is 50 per cent higher (cf. Table III, amount of food and of dry feces), and this increase in the fecal discharge runs parallel to the diminished utilization of the nutriment. The conclusion, therefore, is warranted that in this instance also the digestive activity was somewhat impaired by the short fast. The growth capacity, however, was not affected in this case, the fingerlings having grown more in the 3 weeks following the fast than during the 26 days preceding it. The gain in weight represented 49.8 per cent of the quantity of food per day and kilo, as compared with 42.7 of the earlier period.

Two things must be borne in mind in connection with these experiments: In both instances the evidence clearly indicates that fasting is deleterious to the digestive function of the trout. The difference in effect upon subsequent growth may probably be due to the fact that in Experiment 000.5, the intervening fast was a much more protracted one.

The last experiment on the effect of feeding raw beef heart was performed on the trout which had undergone a preliminary fast (F-1, Table I). In the course of 15 days of the experiment, this trout consumed 27.71 gm. of food with a content of 7.047 gm. of dry matter. Examining the last three columns of Table IV, one

TABLE IV.

*Experiment 000.7. Raw Beef Heart.*

Average temperature.	Period.	Initial or final weight.	Change of weight.	Amount of food.	N intake.	Fat intake.	Dry feces.	Nitrogen in feces.	Fat in feces.	Nitrogen Total N in water eliminated.		N in feces in relation to N intake.		Fat in feces in relation to fat intake.		N in feces in relation to N intake.	
										gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent
17.7	II. 23-II. 26	95.7	—	8.00	0.2880	0.2209	0.0460	0.0050	0.0355	0.1214	0.1264	1.74	16.08	42.2			
14.1	II. 26-III. 1	—	—	3.56	0.1281	0.0983	0.1350	0.0128	0.0090	0.1168	0.1296	10.00	9.16	91.2			
14.7	III. 1-III. 4	—	—	1.95	0.0702	0.0540	0.1280	0.0123	0.0265	0.0918	0.1041	17.52	49.10	130.8			
15.9	III. 4-III. 7	—	—	5.40	0.1943	0.1490	0.1300	0.0113	0.0185	0.1326	0.1439	5.82	12.42	68.3			
16.0	III. 7-III. 10	109.5	—	8.78	0.3162	0.2424	0.1020	0.0148	0.0341	0.1944	0.2092	4.68	14.07	61.5			
15.7	II. 23-III. 10	13.8	+14.5	27.71	0.9968	0.7645	0.6310	0.0562	0.1236	0.6570	0.7132	5.64	16.16	65.9			

notices the high proportion of fat in the feces, and the apparent retention of nitrogen as is shown by the low nitrogen content of excretions passed during the first several days after feeding was resumed. That the retention was purely temporary is seen from the fact that within the next few days the elimination was abnormally high, especially between the 6th and 9th days when it became actually 30.8 per cent more than the nitrogen contained in the food for that period. This delayed elimination of nitrogenous waste points to the possibility that the excretory mechanism of the trout suffered an injury in consequence of fasting, which it required several days, when food was given, to restore to normal functioning.

The similarity of the results obtained in these three experiments is very striking, those of Experiments 000.5 and 000.7 being practically identical.

The dry feces formed a much greater per cent of the dry matter consumed with the food than in any of the previous experiments (8.95 per cent, cf. Table VI). The utilization of the protein during the entire 15 day period is 94.4 per cent, which compares very favorably with the extent of utilization observed in the other experiments. The utilization of the fat which has been reduced to only 83.8 per cent demonstrates again, and more strikingly than in any of the previous experiments, the particularly deleterious influence of fasting upon fat utilization. The reason for this must be looked for in the longer duration of the fast. To explain these facts it may be necessary to assume that a more lasting damage was done to the glandular structures of the animal, the pancreas and the liver, which leads to a defective digestion and absorption of fats. Further investigation of this question would at any rate be desirable.

In spite of the low degree of utilization of the food materials, the index of growth was 50.1; in other words, half of the nutrient material fed has actually gone towards the building up of the body tissues. This result is of much significance, inasmuch as it adds further proof for the idea that neither the utilization of the food in digestion nor the actual quantity consumed determines the extent of the resulting growth of the organism. The trout in this experiment increased in weight at a greater rate than in any other of this series, while actually consuming the smallest quantity of food.

*Feeding Cooked Beef Heart.*

In the practical feeding of fish in hatcheries, the question is frequently asked, Shall the food be fed raw or cooked? The objection to cooked food on the ground that it is not the natural condition of nourishment of the fish, may well be dispensed with. Feeding fish in hatcheries is a problem in domestication, and the merits of a dietary system must be judged not by whether it resembles or deviates from the state of things in nature, that is wild nature, but by the results which can actually be achieved with it. To anticipate what will be brought out in the description of the following experiments, cooked food is neither more nor less utilized than the raw food, though possibly it has less growth-promoting quality. It is not as palatable to the fish as the raw food, and in my experience, the trout ate it much less willingly and in smaller amounts. In only one respect does feeding of cooked food present a decided advantage. On raw food the feces are gelatinous and fairly massive, while those resulting from cooked food are more or less dry and scanty. The feces therefore have no tendency to adhere to the sides of the aquaria, being in well formed compact masses; contamination of the water was never observed when cooked food was fed, and it remained remarkably clear for a number of days.

The food was put up as before, except that it was brought to a quick boil with a small volume of water, the fluid completely drawn off, and the meat packed in stock jars, refrigerated, and analyzed as usual.

Experiment 00.2 (Table V) was made on the same trout which served in Experiment 000.5, and the results of these two experiments are well suited for comparison. The animal was fed on the cooked beef heart for a considerable time to get it thoroughly accustomed to this food before the actual metabolic study was begun. The trout did not relish cooked food, and at best would eat only small quantities. In the 38 days which this experiment lasted, the trout consumed only 23.9 gm. of the food; the feces for this period contained 51 mg. of nitrogen and 34 mg. of fat. These two constituents of the diet were therefore utilized to the extent of 95.7 and 96.1 per cent respectively, which is very close to the values (96.1 and 94.2) which were found on raw meat.



TABLE V.  
*Cooked Beef Heart.*

Experiment No.	Average temperature.	Period.	Initial final weight.	Change Amount of weight, food.	N intake, gm.	Fat intake, gm.	Dry feces, gm.	Nitrogen in feces, gm.	Fat in feces, gm.	N in water elim. (urine), gm.	Total N in relation to N intake, per cent.	Fat in feces in relation to N intake, per cent.	N in water in relation to N intake, per cent.
30.2	°C.			per cent	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
	16.7	IV. 20-V. 30	100.6	—	3.510	0.1776	0.1325	0.0330	—	0.1462	—	—	82.3
	16.3	IV. 30-V. 7	—	—	5.715	0.2801	0.1920	0.0620	—	0.1859	—	—	66.4
	16.3	V. 7-V. 14	108.5	7.81	4.750	0.2404	0.1793	0.0670	—	0.1809	—	—	75.3
	15.4	V. 14-V. 21	—	—	5.175	0.2591	0.1875	0.0860	—	0.2009	—	—	79.9
	16.0	V. 21-V. 28	110.6	2.0	4.760	0.2409	0.1796	0.0554	—	0.1841	—	—	76.4
30.3	16.1	IV. 20-V. 28	10.0	+9.85	23.910	1.1981	0.8710	0.3034	0.0519	0.0340	0.9040	4.33	75.5
	16.2	IV. 30-V. 7	60.9	—	5.370	0.2540	0.1811	0.0420	—	0.1682	—	—	66.4
	16.3	V. 7-V. 14	66.6	9.02	5.120	0.2359	0.1746	0.0462	—	0.1804	—	—	76.5
	15.4	V. 14-V. 21	—	—	4.780	0.2415	0.1803	0.0874	—	0.1935	—	—	80.1
	16.0	V. 21-V. 28	67.4	1.2	4.960	0.2510	0.1871	0.0432	—	0.1839	—	—	73.3
	16.0	IV. 30-V. 28	6.5	+10.66	19.290	0.9818	0.7231	0.2188	0.0401	0.0283	0.7260	4.09	74.0
30.4	16.2	IV. 30-V. 7	56.3	—	1.750	0.0886	0.0690	0.0200	—	0.0699	—	—	78.9
	16.3	V. 7-V. 14	—	—	4.645	0.2350	0.1753	0.0516	—	0.1449	—	—	61.7
	15.4	V. 14-V. 21	60.9	—	4.585	0.2316	0.1728	0.0682	—	0.1583	—	—	68.4
	16.0	V. 21-V. 28	62.6	—	2.380	0.1204	0.0845	0.0238	—	0.0999	—	—	82.9
	16.0	IV. 30-V. 28	6.3	+11.21	13.360	0.6786	0.5039	0.1636	0.0265	0.0378	0.4730	3.92	70.1
	16.8	V. 28-VI. 1	62.6	—	3.150	0.1594	0.1188	0.0615	—	0.1097	—	—	68.9
30.5	15.6	VI. 1-VI. 5	—	—	3.570	0.1806	0.1347	0.0690	—	0.1290	—	—	68.1
	17.8	VI. 5-VI. 9	64.8	—	3.600	0.1822	0.1358	0.0450	—	0.1419	—	—	77.9
	16.5	VI. 9-VI. 13	67.8	—	4.010	0.2029	0.1513	0.0475	—	0.1074	—	—	52.9
	17.4	VI. 13-VI. 17	69.1	—	2.730	0.1381	0.1030	0.0688	—	0.1359	—	—	98.4
	16.8	V. 28-VI. 17	6.5	+8.31	17.060	0.8632	0.6436	0.2918	0.0763	0.0479	0.6179	8.84	71.6
	16.4	IV. 30-VI. 17	12.8	+22.74	30.421	1.5308	1.1475	0.4554	0.1028	0.0857	1.0909	6.68	70.9

The per cent of nitrogen and fat in the dry feces is much greater than on a diet of raw meat, and this condition is very general as can be seen by comparing the data in Columns 5 and 6 of Table VI. This very high percentage, however, is of no significance, being merely due to the fact that the feces are more compact and dry when the trout are kept on the cooked meat régime. It is more significant that the amount of dry feces presents the same proportion of the dry matter of the consumed food (3 to 4 per cent) no matter whether the food has been cooked or not.

TABLE VI.

Experiment No.	Duration.	Nature of food.	Ratio of dry feces to dry matter of food.	Dry feces.		Utilization.		Average intervals between feedings.	Food consumed.	Increase in body weight.	Index of growth.
				Nitrogen.	Fat.	Protein.	Fat.				
				per cent	per cent	per cent	per cent		Per kg. and per 24 hrs.		
	days		per cent	per cent	per cent	per cent	per cent	hrs.	gm.	gm.	
000.5(a)	32	Raw beef heart.	4.99	10.0	10.0	96.1	94.2	48	37.5	17.40	46.5
000.5(b)	15	"	7.80	9.6	9.3	94.8	91.7	48	26.7	9.73	36.5
000.6(a)	26	"	3.52	9.1	6.4	97.5	96.2	48	25.0	10.67	42.7
000.6(b)	21	"	6.42	10.0	10.0	95.4	91.8	48	27.0	13.45	49.8
000.7	15	"	8.95	8.9	17.9	94.4	83.8	48	18.0	17.02	50.1
00.2	38	Cooked beef heart.	3.4	19.1	12.7	95.7	96.1	41	6.0	2.50	41.7
00.3	28	"	3.0	20.9	14.6	95.9	96.1	34	10.78	3.63	33.7
00.4	48	"	3.7	22.6	18.8	95.7	92.5	36	10.11	4.25	42.0

The results of Experiment 00.3 (Table V) are essentially the same, though this trout has shown a somewhat smaller increase in weight. Experiment 00.4 which lasted 48 days was made on a trout which had been fasting a week before the experiment commenced. The protein of the food was utilized as usual—95.7 per cent, but only 92.5 per cent of the fat was utilized. This further confirms the point emphasized already in this paper, of the defective utilization of fat by trout that have undergone even a brief fast. The amount of food this trout ingested per day and kilo of body weight was 10.11 gm. The daily increment in

weight per kilo was 4.25 gm.; in other words, the growth index was 42. When we compare the relative values of cooked and raw meat as the diet for trout, we can indicate the following advantages of the latter: its greater palatability and greater growth-promoting quality. This can be seen at once, when the average growth index of Experiments 000.5 to 000.7, which is 45, is compared with the same of Experiments 00.2 to 00.4, which is only 39. This might perhaps be objected to on the ground that the difference in the body weight increments is due rather to the great difference in the quantity of food which the trout has consumed in these two series of experiments. It must be recalled, however, that the growth index does not furnish information as to the actual or absolute increment in body weight, but indicates the fraction of the nutrient material which has become permanently incorporated as a part of the organism. The two kinds of food have been apparently ample in amount to insure not only maintenance, but a further increase in weight, but in the case of the cooked beef heart a smaller relative increase in body weight was secured. It is possible that the lesser effect of the latter in producing growth was due to the fact that in the process of boiling, some of its water-soluble growth-stimulating substances have been dissolved out. The fact that the food which has been boiled and thus lost a large part of its water content was as well utilized by the trout as raw meat, suggests that it would be practicable to utilize thoroughly desiccated food as trout diet. The food soaked in water before feeding would probably retain its gustatory quality, as well as its growth-promoting quality. This might be an excellent method for preparing on a large scale and distributing the food for trout hatcheries. Experiments, which were planned with a view of studying the metabolic value of such desiccated foods, remained unperformed owing to the unexpected interruption of the investigation.

#### *Feeding Beef Liver.*

Beef or pig liver is one of the staple diets in trout hatcheries, and a series of experiments was started to study its nutritive value. The liver was freed from all blood vessels and ducts, and the parenchyma alone was frozen solid and ground in a meat

grinder to a fine pulp. The food was then kept in the refrigerator and analyzed as usual. Unfortunately it was impossible to feed this food directly from forceps as was done with the beef heart. It was necessary instead to throw a quantity of food into the aquaria and leave it there for half an hour. The trout was then removed to another aquarium with fresh water, in which the excreta were collected, while the unconsumed food was collected over glass wool in a large filtering tumbler. The filtrate was made up to a definite volume, of which aliquot portions were used to determine the nitrogen, fat, and sugar that were washed out from the food by the water. The solid residue was dried at 100°, powdered, and the composite sample for the entire experimental period was analyzed in the usual way.

Knowing the quantity of food thrown into the aquarium, the amounts of nitrogen, fat, and carbohydrate were computed from the analytical data pertaining to the particular food sample. From this were subtracted the amounts of nitrogen, fat, and carbohydrate, found both in the water and in the solid residue. The consumed amount of each constituent was then determined by the difference.

In his study of the digestion of fish, Knauthe<sup>1</sup> made use of this method exclusively. Apart from the disadvantage which it shares with all indirect methods, the method is time-consuming and presents so many unforeseen difficulties as to render the results at times of no value.

In the matter of recovering the fat, Knauthe's procedure, though very simple, consisting merely in extracting an aliquot portion of the water with ether, on closer examination proves of little or no value. Knauthe has not performed blank experiments to determine how closely the recovered amount corresponded with that washed out by the water; he therefore had no occasion to be apprehensive about the acceptability of his results.

As was pointed out above, I considered the performance of blank experiments essential, and have relied on these in deciding whether or not the analytical procedure in use was adequate. By following Knauthe's method, I found that the results were so widely different from the expected values as to make them worthless.

<sup>1</sup> Knauthe, K., *Z. Fischerei*, vi, 1898, 139.

It will be noted further that in all the experiments the animals were allowed to remain in the feed jars half an hour, after which they were removed to fresh water. Knauthe on the other hand, allowed his animals to remain in the aquaria containing food for a long time, and separated mechanically the feces from the solid food particles. It need hardly be pointed out that such a procedure is too crude to warrant great confidence in the significance of experimental results. The bacterial growth in suspensions of nutrient material would be sufficient to vitiate the results. In a number of blank experiments a weighed quantity of liver pulp was put in the aquaria (without trout), left there half an hour as usual, then the solid residue and the filtered water were analyzed. In the case of the nitrogenous material and the sugar, the recovery was complete within less than 1 per cent, but in the matter of the fat, as determined by direct extraction of the water with ether, the values were usually so low as to render the data of no importance. The need of a different and better method was keenly appreciated, and attempts in this direction were started. The main difficulty to be remembered, was that a relatively small quantity of fat had to be recovered quantitatively from a large bulk of water.

All the determinations were in duplicate. The nitrogen was determined by the Kjeldahl method, and one-tenth portions of the entire water were used for the analysis. The sugar was determined by Bertrand's method in one-quarter portions. The sugar analysis was made on the perfectly clear filtrate, which was obtained by treating the water with aluminum cream to free it of the protein. On extracting this clear filtrate with ether, I invariably found that there was no fat present. This suggested a method of determining the fat which is evidently carried down with the aluminum precipitate. By drying this voluminous gelatinous precipitate, powdering it, and extracting it with ether, it was hoped that accurate estimate of the fat suspended in the water would be obtained. While this method, or some similar modification of it, has good possibilities, a number of difficulties were encountered in its practical application, which were not entirely removed before this work was interrupted, and the matter of utilization of the fat from liver was not touched in this investigation.



The feeding of liver has not met with success in my hands. A number of circumstances have probably conspired to make these experiments less definite than those of the preceding series. Many experiments failed, owing to the death or ill condition of the trout. Three experiments are recorded in Table VII, and these will perhaps throw light on the question of liver as a trout food. It will be seen that the trout did not grow as well as those fed on beef heart; indeed, one specimen lost 8.5 gm. The utilization of protein (nitrogen) was very low (90.5 to 83.6 per cent). The utilization of glucose likewise shows wide fluctuation, from 96.9 to 86.9 per cent; that of the fat ranged about 90 per cent, but as the analytical data were not entirely reliable, these results have not been included in the table.

Before concluding, one other point should be mentioned. In the course of the half hour during which the liver pulp remained in the aquaria, as much as 60 to 75 per cent of its nitrogenous material has been dissolved by the water. It is a conservative estimate that 50 per cent of the fat was washed out, and none of the glucose was left in the residue, the glucose having been entirely dissolved out by the water. This happened in spite of the fact that the food was contained in a limited quantity of water, agitated only by the swimming movements of the trout.

In hatcheries where the trout are kept in rapid streams of water, it is questionable whether a food, which will so easily give up its components to the water, is a particularly valuable diet. Its inferiority to beef heart as a dietary article for trout is amply demonstrated by the results of the experiments recorded here.

#### SUMMARY.

A study of the utilization of the fundamental food materials—protein, fat, and carbohydrate—has been made on trout. The per cent of utilization under normal circumstances was found to be high. Fasting has a deleterious effect on absorption, particularly of the fat.

Fasting trout eliminate 80 to 90 mg. of nitrogen per day and per kilo. But if the fast has been preceded by a liberal diet, the nitrogen elimination for the first few days may greatly exceed

this. About one-fifth of the loss in body weight is at the expense of protein.

A comparison of diets consisting of raw and cooked beef heart showed that both are equally well utilized in the organism of the trout, but a greater gain in body weight is secured from the former. Increase in weight does not bear a direct relation to the absolute quantity of food consumed.

This study fails to justify the use of beef liver as a dietary article for trout.





# ANIMAL CALORIMETRY.

## FIFTEENTH PAPER.

### FURTHER EXPERIMENTS RELATIVE TO THE CAUSE OF THE SPECIFIC DYNAMIC ACTION OF PROTEIN.

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## INTRODUCTION.

The work accomplished in this laboratory<sup>1</sup> has shown that certain amino-acids, such as glycocoll and alanine, when given to a dog cause a great increase in the heat production of the animal, and that this increase in heat production is proportional to the number of hydroxy-acid molecules, glycollic or lactic, which can be liberated on the deamination of glycocoll and alanine. Since no increase in metabolism was caused by the ingestion of glutamic acid, it was concluded that the processes of deamination or of urea formation played no rôle in the increased heat production. The proof that amino-acids themselves are not stimuli of metabolism lies in the fact that that quota of ingested protein which is absorbed as amino-acids and rebuilt into new body pro-

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<sup>1</sup> Lusk, G., *J. Biol. Chem.*, 1912-13, xiii, 155; 1915, xx, 555.

tein exerts no specific dynamic action; and the proof that the increased heat production does not come from the oxidation of the materials themselves is demonstrated by the observation that when glycocholl and alanine are given in phlorhizin glycosuria they are transformed into glucose without oxidation, are eliminated in the urine, and yet exert a stimulating effect on the metabolism.

In 1915 Grafe<sup>2</sup> published a series of experiments upon rabbits, dogs, and men relative to the specific dynamic action of protein, some of which experiments, especially those concerning glycocholl and alanine, confirmed, while others contradicted Lusk's researches, and yet others were self-contradictory. For example, Grafe found in a dog that the absorption of oxygen was increased between 16 and 17 per cent during  $3\frac{1}{2}$  hours following the ingestion of 50 gm. of glutamic acid (as a sodium salt), whereas Lusk had found no increase after giving 20 gm. Furthermore, Grafe reported that after giving 67.5 gm. of asparagine to a man the metabolism was uninfluenced during successive periods, respectively, of  $4\frac{1}{2}$ , 4, and 2 hours duration, although he found an increase of 11 per cent in the oxygen absorption after giving 47 gm. to another man. This last experiment is open to the following criticisms: (1) The man was in the hospital undergoing treatment for syphilis. (2) The basal metabolism was determined 5 days before the day of asparagine ingestion, during which interval the patient is stated to have increased in weight from 50.2 to 51.5 kilos. (3) The respiratory quotient of the 10 hour interval of the basal metabolism period was 0.95, notwithstanding the fact that this period began 12 hours after taking food. Such a respiratory quotient could scarcely have been obtained had the patient been given sugar in large excess on the day previous.<sup>3</sup> This leads to the suspicion that the oxygen determination was too low. The carbon dioxide excretion rose only 5 per cent as a result of the asparagine ingestion.

This questionable determination of the basal metabolism throws doubt upon reported increases in the metabolism of this man, of 13 per cent after giving 28 gm. of phenylalanine; of 14 per cent

<sup>2</sup> Grafe, E., *Deutsch. Arch. klin. Med.*, 1915-16, cxviii, 1.

<sup>3</sup> Benedict, F. G., Emmes, L. E., and Riehe, J. A., *Am. J. Physiol.*, 1910-11, xxvii, 383.

after giving 12 gm. of ammonium chloride; and of 22 per cent after giving 42 gm. of acetamide.

On the basis of these and other experiments Grafe comes to the conclusion that the specific dynamic action of protein is largely due to the liberation of the  $\text{NH}_2$  radicle of amino-acids. His table showing this relation in man is inconclusive. However, the table showing the relation between nitrogen intake and extra heat production in the dog may be quoted in part as follows:

Substance given.	N	Amount in food.	N content.	Increase in calories per gm. of N ingested.	Author.
				Calories N	
	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>		
Glycocoll.....	18.7	25	4.66	4.48	Lusk.
Alanine.....	15.7	20	3.14	4.00	"
".....		50 (?)	7.85 (?)	4.5 (?)	Grafe.
Leucine.....	10.7	20	2.14	2.9	Lusk.
Glutamic acid.....	9.5	20	1.90	0	"
" ".....		50 (?)	4.75 (?)	5.6 (?)	Grafe.
Tyrosine.....	7.7	20	1.55	5.0	Lusk.
100 gm. meat.....	3.0	100	3.00	4.8	"

The interrogation points after Grafe's work, which are found in the above tabulation, are in his original table and are due to the fact that the materials given to the dog were in small part vomited.

It is evident that the main disagreement in the results obtained in the two laboratories lies in the difference in the outcome of the two experiments in which glutamic acid was ingested. The validity of Lusk's conclusions has also been challenged by others<sup>4</sup> and it seemed desirable to subject the matter to further investigation. As no glutamic acid was available, aspartic acid was employed in its stead. Asparagine was also given. Since this latter substance and glycocoll both contain 18.7 per cent of nitrogen, it would follow from Grafe's hypothesis that each should exert the same specific dynamic action. Also, the theoretically interesting administration of acetamide was worthy of

<sup>4</sup> Notably by Dr. H. H. Mitchell in private correspondence.

repetition and, in addition to this, experiments were instituted on the ingestion of succinic acid, which Ringer<sup>5</sup> and coworkers deem to be an intermediary metabolite of glutamic acid. *The following experiments wholly failed to substantiate Grafe's conclusions.*

#### EXPERIMENTAL.

The same experimental procedure was employed as in the work described in former papers.<sup>6</sup> The ingestion of materials followed about 18 hours after giving the usual "standard diet."

#### *Urinary Nitrogen.*

*Dog XVI.*—After giving 10 gm. of aspartic acid dissolved in hydrochloric acid, the nitrogen excretion during the first 3 hours and 37 minutes amounted to 0.25 gm. per hour in contrast with not more than 0.1 gm. per hour during periods of the determination of the basal metabolism. The administration of *hydrochloric acid* alone slightly increased the nitrogen elimination above the basal level to 0.12 gm. per hour from 0.09 gm. per hour.

*Dog XVII.*—The more striking details of the urinary analyses in this dog may be presented in Table I. The results after the ingestion of materials were obtained from the urine of the first 3 to 4 hours thereafter.

It is evident from Table I that *asparagine* and *ammonium citrate* were both freely absorbed and converted into urea, whereas *acetamide*, though absorbed, did not increase the urea in the urine. The ingestion of *succinic acid* increased the total nitrogen in the urine as did hydrochloric acid in Dog XVI.

*Computation of the Respiratory Metabolism.*—A method for the computation of the metabolism of ingested glycocholic acid has been described in a previous paper of this series.<sup>6</sup> It was based on the fact that when glycocholic acid is given in phlorhizin glycosuria 22 per cent of it is eliminated per hour as extra sugar in the urine during the 2nd and 3rd hours and a lesser quantity thereafter. This was accepted as a better guide to the rapidity of the normal me-

<sup>5</sup> Ringer, A. I., Frankel, E. M., and Jonas, L., *J. Biol. Chem.*, 1913, xiv, 539.

<sup>6</sup> Lusik, *J. Biol. Chem.*, 1915, xx, 555.

tabolism than the nitrogen excretion of the corresponding hours, since this excretion is known to lag considerably behind the formation of urea in the body.

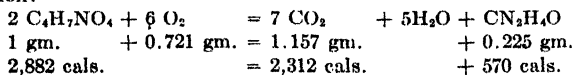
In the present series of experiments it has been arbitrarily assumed that 50 per cent of the ingested aspartic acid, asparagine, and succinic acid were metabolized during the 2 hour period which included the 2nd and 3rd hours after the ingestion of the

TABLE I.

Date.	Experi- ment No.	Food.	Total N.	Urea N.	NH <sub>2</sub> N
1918			gm.	gm.	gm.
Feb. 11	16	Basal.	0.130		
" 12	17	Asparagine, 15 gm.	0.170	0.157	0.010
" 18	21	" 15 "	0.182	0.167	0.011
" 21	23	Lard, 25 gm.	0.117	0.101	0.010
" 26	25	Asparagine, 15 gm.	0.219	0.207	0.009
" 28	26	Basal.	0.115		
Mar. 4	28	"	0.106		
" 7	29	Succinic acid, 11.2 gm.	0.140		
" 8	30	Basal.	0.101		
" 9	31	Succinic acid, 11.2 gm.	0.147		
" 12	32	Basal.	0.097		
" 14	34	Acetamide, 12 gm.	0.190	0.070	0.020
" 15	35	Basal.	0.135		
Jan. 16	5	Acetamide, 12 gm.	0.180	0.064	0.016
" 17	6	Basal.	0.093		
" 25	11	"	0.110		
" 29	12	Ammonium citrate, 6 gm.	0.253	0.179	0.025

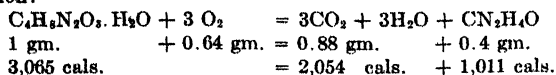
substances. The computations were based on the following considerations.

*Aspartic Acid*.—Calories per gm. = 2,882. The oxidative physiological reaction:



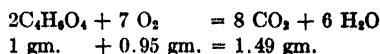
Physiological values: R. Q. = 1.17, 1 gm. = 2,312 calories, 1 liter O<sub>2</sub> = 4.578 calories.

*Asparagine*.—Calories per gm. = 3,065. The oxidative physiological reaction:



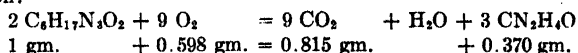
Physiological values: R. Q. = 1, 1 gm. = 2,054 calories, 1 liter O<sub>2</sub> = 4.562 calories.

*Succinic Acid*.—Calories per gm. = 3,026. The oxidative physiological reaction:



Physiological values: R. Q. = 1.14, 1 liter  $\text{O}_2$  = 4.550 calories.

*Ammonium Citrate*.—Calories unknown. The oxidative physiological reaction:



Physiological value of R. Q. is unity.

### *Influence of Aspartic Acid and of Hydrochloric Acid.*

10 gm. of aspartic acid dissolved in 200 cc. of a solution containing 0.4 per cent of hydrochloric acid and 0.2 gm. of Liebig's extract of beef were warmed to a temperature of 50°C. and administered to a dog through a stomach sound. The results were compared with those obtained when a like amount of hydrochloric acid was given, and also with the *basal metabolism* when no food was given.

The results may be summarized in Table II, which shows the influence on the heat production of Dog XVI of 10 gm. of aspartic acid in 200 cc. of 0.4 per cent HCl solution and of the latter alone. Results are in 2 hour periods which began 1 hour after the ingestion of the material.

TABLE II.

Date.	Experiment No.	Food.	R. Q.	Calories.	
				Indirect.	Direct.
1917					
Nov. 12	10	Basal.	0.78	40.67	40.65
" 14	11	HCl.	0.78	42.48	41.38
" 19	12	Aspartic acid + HCl.	0.85	42.49	40.54
" 21	13	Basal.	0.76	38.94	38.84
" 23	14	"	0.72	41.28	40.89
" 26	15	Aspartic acid + HCl.	0.87	42.05	39.22
" 27	16	Basal.	0.76	40.23	37.36
" 28	17	HCl.	0.80	20.13*	39.46
Dec. 5	18	Basal.	0.78	38.78	37.40

\* 1 hour only.

The administration of another dose of hydrochloric acid resulted in the death of the animal, with all symptoms of acid poisoning, a regrettable accident, since the observation on Nov. 28 was incomplete on account of a slight leak of oxygen during the 2nd hour.

The higher respiratory quotients on the days of aspartic acid ingestion conform with expectations.

The evidence found in Table II may be further summarized as follows so as to indicate the influence of the two factors, aspartic acid and hydrochloric acid:

No. of experiments.	Food.	Calories.			
		Indirect.	Increase above basal.	Direct.	Increase above basal.
5	Basal.	40.0		39.0	
2	HCl.	42.5*	2.5	40.4	1.4
2	Aspartic acid + HCl.	42.7	2.7	40.0	1.0

\* One experiment only.

It is evident from this tabulation that, *although the administration of hydrochloric acid may cause a slight increase of 2.5 calories (6 per cent) in the basal metabolism, the simultaneous administration of 10 gm. of aspartic acid will cause no further increase.*

If one compares these results with computations of the first 2 hours of the recorded metabolism after giving 5.5 gm. of glycocoll to Dog III,<sup>7</sup> one arrives at the following table:

Dog.	Experiment No.	Food.	N in food.	Calories.	
				Indirect.	Increase.
III	5, 6, 21	Basal.		39.6	
	13	Glycocoll, 5.5 gm.	1.03	43.3	3.7
	9, 10, 14	Alanine, 5.5 gm.	0.87	43.0	3.4
XVI	11	HCl.		42.5	
	12, 15	Aspartic acid, 10 gm., + HCl.	1.05	42.7	0.2

*These experiments with aspartic acid corroborate the former work with glutamic acid and indicate that the two dibasic acids occurring*

<sup>7</sup> Lusk, *J. Biol. Chem.*, 1915, xx, 568.



*in protein exert no specific dynamic action upon metabolism.* The increase of 17 per cent in the metabolism of a dog during the first 3½ hours after giving 50 gm. of glutamic acid, as observed by Grafe, might be due to the large number of metabolites introduced into the circulation—just as 50 gm. of glucose would effect the same result.

### *Influence of Asparagine.*

Owing to the insolubility of asparagine, it was given mixed with 25 gm. of fat. When glutamic acid was given with lard to a phlorhizinized dog<sup>8</sup> the former was absorbed and converted into glucose within 12 hours after its administration, and this

TABLE III.

Date.	Experiment No.	Food.	R. Q.	N in urine.	Calories.	
					Indirect.	Direct.
1918				gm.		
Jan. 25	11	Basal.	0.83	0.220	43.09	37.53
Feb. 11	16	Lard, 25 gm.	0.82	0.260	45.78	
" 12	17	" 25 " + asparagine, 15 gm.	0.92	0.340	42.20	44.39
" 18	21	" 25 " + " 15 "	0.88	0.364	45.06	43.59
" 21	23	" 25 "	0.80	0.234	45.29	42.16
" 26	25	" 25 " + asparagine, 15 gm.	0.88	0.438	45.31	42.19
" 28	26	Basal.	0.83	0.230	43.09	38.60

older work suggested the present procedure. In the present series of experiments evidences of the absorption of asparagine are found in the increased quantity of urinary nitrogen and in the consistently higher respiratory quotients obtained on the days of ingestion of asparagine, which substance gives a respiratory quotient of unity.

Table III presents the main results of the investigation of the influence on the heat production of Dog XVII of 15 gm. of asparagine given with 25 gm. of lard, and of the latter alone. Results are in 2 hour periods which began 1 hour after the ingestion of the substance.

<sup>8</sup> Lusk, *Am. J. Physiol.*, 1908, xxii, 174.

It is evident from Table III that the addition of 15 gm. of asparagine containing 2.8 gm. of nitrogen to a diet containing a small amount of fat has no effect whatever upon the heat production of a dog. It was established by Murlin and Lusk<sup>7</sup> that when glycocoll and fat are absorbed together the increase in the heat production amounts to the sum of the increases which either substance alone would have produced. The increase in the metabolism of a dog during the 2nd and 3rd hours after the ingestion of 20 gm. and of 10 gm. of glycocoll, as determined by Lusk,<sup>7</sup> may thus be summarized:

*Dog III.*

Glycocoll.	N in food.	Increase in metabolism during 2 hrs.
gm.	gm.	calories
10	1.9	7.6
10	1.9	8.8
20	3.7	14.6
20	3.7	13.8

*It is evident, therefore, that asparagine and glycocoll, which contain the same percentage quantity of nitrogen, behave very differently in metabolism, the first being without specific dynamic action and the other exerting the most powerful specific dynamic action of any of the amino-acids contained in protein which have thus far been tested. Therefore, the specific dynamic action of protein is not due to the  $\text{NH}_2$  radicle of the amino-acid molecule.*

#### *Influence of Succinic Acid.*

If the dibasic acid, glutamic acid, is oxidized at the  $\alpha$ -amino carbon position with cleavage of carbon dioxide, succinic acid would result. If in accordance with Ringer's<sup>6</sup> conception, this is the first intermediary product of the metabolism of glutamic acid, then the ingestion of this substance should show no pronounced effect upon the metabolism. That such is the case is borne out in Table IV, which shows the influence of 11.8 gm. of succinic acid dissolved in 200 cc. of water upon the heat production of Dog XVII, periods of 2 hours.

<sup>7</sup> Murlin, J. R., and Lusk, G., *J. Biol. Chem.*, 1915, xxii, 15.

It is evident that 11.8 gm. of succinic acid, which is the amount that might have been derived from 14.7 gm. of glutamic acid, has no appreciable influence upon the metabolism of a dog.

Since succinic acid would produce a respiratory quotient of 1.14, higher quotients are observed on the days of its ingestion than on the days of basal metabolism.

TABLE IV.

Date.	Experiment No.	Food.	R. Q.	N in urine.	Calories.	
					Indirect.	Direct.
1918				gm.		
Feb. 28	26	Basal.	0.83	0.230	43.29	38.60†
Mar. 4	28	"	0.81	0.212	43.83	39.74†
" 7	29	Succinic acid, 11.8 gm.	0.87	0.280	43.98	44.28
" 8	30	Basal.	0.81	0.202	41.65	37.86†
" 9	31	Succinic acid, 11.8 gm.	0.93	0.147*	21.07*	20.60*
" 12	32	Basal.	0.80	0.194	41.21	39.48

\* 1 hour only.

† First hour low. It is probable that the divergence between the indirect and the direct calorimetry, was due to the fact that the iron work of the calorimeter was not fully warmed at the beginning of the experiment. The room was allowed to get cold each night.

### *Influence of Acetamide, 12 Gm.*

Acetamide is a chemical isomer of glycocholl and, according to Grafe, is deaminized after ingestion by a man and increases the heat production. In the dog it is neither deaminized nor does it increase the heat production. The latter fact appears from Table V, which shows the effect of 12 gm. of acetamide (= 2.04 gm. N) dissolved in 200 cc. of water upon the heat production of Dog XVII. Results are in 2 hour periods which began 1 hour after the ingestion of the substance.

TABLE V.

Date.	Experiment No.	Food.	R. Q.	N in urine.	Calories.	
					Indirect.	Direct.
1918				gm.		
Mar. 12	32.	Basal.	0.80	0.194	41.21	39.48
" 14	34	Acetamide, 12 gm.	0.87	0.380	39.83	38.03
" 15	35	Basal.	0.88	0.270	40.82	

*Influence of Citrate of Ammonium, 6 Gm.*

When 6 gm. of citrate of ammonium, containing 1.04 gm. of nitrogen, were given to the dog in one experiment there was no appreciable increase in the metabolism. As the caloric value of the substance was unknown, the exact heat production could not be estimated, but using the ordinary procedure, the indirect heat production for 2 hours may be calculated as follows:

Date.	Experiment No.	Food.	Calories.
1918			
Jan. 25	11	Basal.	43.09
" 29	12	Ammonium citrate, 6 gm.	44.76

This rise of 1.65 calories (which is within the limits of error in the determination of the basal metabolism of a dog) may be compared with one of 3.7 calories already cited as having been produced after giving 5.5 gm. of glycocoll containing 1.03 gm. of nitrogen.

## SUMMARY.

It has been shown that 200 cc. of 0.4 per cent of *hydrochloric acid* may slightly increase the metabolism of a dog; that *aspartic acid* and *asparagine* exert no specific dynamic action upon metabolism in the sense that glycocoll and alanine do; that *succinic acid* (a possible intermediary metabolite of glutamic acid) is without the power to increase the heat production, which accords with the previously determined behavior of glutamic acid; that *acetamide* is not deaminized by the dog nor does it increase the heat production of the animal.

The conclusion formerly expressed that the processes of deamination and urea formation have nothing to do with the specific dynamic action of protein is upheld by these later researches.

Some assistance was given in accomplishing this work by second year medical students: Messrs. T. M. French, H. B. Adams, M. M. Harris, A. G. Davidson, W. I. Galland, and H. E. Montero.

TABLE VI.—General

Date.	Experiment No.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	H <sub>2</sub> O	Urine, basal N.	Non-protein R.Q.	Calories.		
									Protein.	Amino-acid.	Non-protein.
1917		p.m.	gm.	gm.		gm.	gm.				
Nov. 12	10	2.40-4.40	13.13	12.25	0.78	21.12	0.168	0.78	4.46		36.21
" 14	11	2.56-4.56	13.81	12.83	0.78	20.79	0.228	0.80	6.04		36.44
" 19	12	3.11-5.11	15.37	13.06	0.86	21.45	(0.168)	0.72	4.46	11.56	26.47
" 21	13	2.36-4.36	12.40	11.79	0.76	19.39	0.160	0.76	4.24		34.70.
" 23	14	2.33-4.33	12.59	12.65	0.72	24.94	0.200	0.71	5.30		35.98
" 26	15	3.06-5.06	15.75	13.12	0.87	18.98	(0.200)	0.75	5.30	11.56	25.99
" 27	16	2.32-4.32	12.71	12.21	0.76	15.29	0.192	0.75	5.10		35.13
" 28	17	3.10-4.10	6.67	6.06	0.80	9.94	0.123	0.80	3.26		16.87
Dec. 5	18	2.03-4.03	12.61	11.68	0.79	18.47	0.180	0.78	4.78		34.00

TABLE VII.—General

Date.	Experiment No.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	H <sub>2</sub> O	Urine, basal N.	Non-protein R.Q.	Calories.		
									Protein.	Special substance.	Non-protein.
1918		p.m.	gm.	gm.		gm.	gm.				
Jan. 25	11	2.00-4.00	14.63	12.86	0.83	17.26	0.220	0.83	5.84		37.25
" 29	12	2.08-4.08	15.82	13.20	0.87	19.20	(0.220)*	0.88	5.84		38.92
Feb. 11	16	3.03-4.03	15.37	13.71	0.82	17.03	0.260	0.82	6.90		38.88
" 12	17	1.16-3.16	16.07	12.80	0.92	18.59	(0.260)	0.88	6.90	15.40	19.90
" 18	21	2.22-4.22	16.58	13.71	0.88	17.58	(0.110)	0.82	5.84	15.40	23.82
" 21	23	1.49-3.49	15.01	13.60	0.80	15.18	0.234	0.80	6.20		39.09
" 25	25	1.25-3.25	16.73	13.78	0.88	17.33	(0.234)	0.83	6.20	15.40	23.71
" 28	26	1.19-3.19	14.78	12.90	0.83	17.00	0.230	0.84	6.10		37.19
Mar. 4	28	12.52-2.52	14.64	13.13	0.81	16.38	0.212	0.82	5.62		38.21
" 7	29	12.29-2.29	16.99	13.67	0.90	23.85	0.280	0.71	7.42	17.85	18.71
" 8	30	12.36-2.36	13.93	12.47	0.81	16.43	0.202	0.81	5.36		36.29
" 9	31	12.13-1.13	8.27	6.54	0.95	9.06	(0.147)	0.73	3.90	8.92	8.25
" 12	32	12.40-2.40	13.63	12.37	0.80	16.76	0.194	0.80	5.14		36.07
" 14	34	12.23-2.23	14.00	11.76	0.87	17.43	(0.194)	0.87	5.14		34.69
" 15	35	12.13-2.13	14.56	12.07	0.88	16.18	0.135	0.89	7.16		33.66

\* Nitrogen values in parentheses are assumed from nearby records of the basal

*Summary of Dog XVI.*

Calories.		Body temperature.			Morn- ing weight.	Behavior of dog.	Food.
Total calcu- lated.	Total found.	Start.	End.	Differ- ence.			
		°C.	°C.	°C.	kg.		
40.67	40.65	38.48	38.29	-0.19	10.5	Quiet.	Basal.
42.48	41.38	38.78	38.47	-0.31	10.8	"	2 p.m., 200 cc. 0.4 per cent HCl.
42.49	40.54	38.47	38.26	-0.21	10.8	"	2.20 p.m., 10 gm. aspartic acid + 200 cc. 0.4 per cent HCl + 0.2 gm. meat extract.
38.94	38.84	38.56	38.45	-0.11	10.6	"	Basal.
41.28	40.89	38.60	38.36	-0.24	10.7	"	"
42.85	39.22	38.51	38.30	-0.21	10.9	"	2.10 p.m., aspartic acid as above.
40.23	37.36	38.67	38.54	-0.13	10.7	"	Basal.
20.13	18.34	38.69	38.49	-0.20	10.7	"	2 p.m., 200 cc. 0.4 per cent HCl.
38.78	37.40	38.35	38.45	-0.10	10.9	"	Basal.

*Summary of Dog XVII.*

Calories.		Body temperature.			Morn- ing weight.	Behavior of dog.	Food.
Total calcu- lated.	Total found.	Start.	End.	Differ- ence.			
		°C.	°C.	°C.	kg.		
43.09	37.53	38.35	38.17	-0.18	14.7	Quiet.	Basal.
44.76	44.24	38.44	38.37	-0.07	14.9	"	12 m., 12.30, and 1 p.m., in 3 por- tions, 6 gm. ammonium citrate in 200 cc. water.
45.78					14.7	"	1 p.m., 25 gm. lard.
42.20	44.39	38.38	38.43	+0.05	14.7	"	12.35 p.m., 15 gm. asparagine + 25 gm. lard.
45.06	43.59	38.51	38.45	-0.06	14.8	"	1.35 p.m., asparagine as above.
45.29	42.16	38.28	38.34	+0.06	14.8	"	1.10 p.m., 25 gm. lard.
45.31	42.19	38.30	38.36	+0.06	14.8	"	12.35 p.m., asparagine as above.
43.09	38.60	38.30	38.24	-0.06	14.8	"	Basal.
43.83	39.74	38.44	38.27	-0.17	14.9	"	"
43.98	44.28	38.58	38.36	-0.22	15.1	"	11.40 a.m., 11.8 gm. succinic acid in 200 cc. water at 39°C.
41.65	37.86	38.30	38.09	-0.21	15.0	"	Basal.
21.07	20.60	38.14	38.20	+0.06	15.1	"	11.30 a.m., succinic acid as above.
41.21	39.48	38.36	38.30	-0.06	15.0	"	Basal.
39.83	38.03	38.08	37.91	-0.17	15.1	"	11.45 a.m., 12 gm. acetamide in 200 cc. water.
40.82	36.98	38.26	38.19	-0.07	15.0	"	Basal.

values.



## THE SULFUR REQUIREMENT OF THE RED CLOVER PLANT.\*

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### PLATE 1.

(Received for publication, September 23, 1918.)

Several years ago, Hart and Peterson<sup>1</sup> called attention to the apparent deficiency of sulfur in certain types of soil, as related to the demands made upon this element by some species of agricultural plants. More recently, Hart and the writer<sup>2</sup> have reported increased production of dry matter by plants of various species which had received calcium and sodium sulfates in soil cultures conducted in a greenhouse. This effect was particularly striking in the case of common red clover (*Trifolium pratense*), which gave a greater response to calcium sulfate than to sodium sulfate. Other investigators, notably Shedd,<sup>3</sup> have obtained similar beneficial effects from sulfates with soil cultures. Pitz<sup>4</sup> devoted special attention to the effects of calcium sulfate upon the growth of red clover. He found that the development of red clover bacteria, as well as of the young host plant, was stimulated by this source of sulfur.

The present paper deals with the response of the common red clover plant to different forms and planes of sulfur supply, under conditions better controlled than the ordinary soil culture. As nutrient medium, use has been made of the well known nutrient

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

<sup>1</sup> Hart, E. B., and Peterson, W. H., *J. Am. Chem. Soc.*, 1911, xxxiii, 549.

<sup>2</sup> Hart, E. B., and Tottingham, W. E., *J. Agric. Research*, 1916, v, 233.

<sup>3</sup> Shedd, O. M., *Kentucky Agric. Exp. Station, Bull.* 174, 1914, 595.

<sup>4</sup> Pitz, W., *J. Agric. Research*, 1916, v, 771.



solution of Knop,<sup>5</sup> with a total salt concentration of 0.2 per cent, by volume. It is quite possible that this solution may be improved for the growth of clover by readjusting its salt content, as Shive and also the writer have found with wheat,<sup>6</sup> but, until such readjustment should be tested, it seemed to be as suitable for the present purpose as any other nutrient solution.

The methods followed with the water cultures were those previously employed by the writer,<sup>6</sup> except that the seeds were germinated in pure sand, instead of over water, until the seedlings were large enough for fixing in the culture vessels. About 10 days were required for this stage of development. In order to avoid the high hydrogen ion content which would result from the use of  $\text{KH}_2\text{PO}_4$  as the only source of phosphorus, this salt and  $\text{K}_2\text{HPO}_4$  were added to the solution in equimolecular proportions, each in one-half the usual molecular concentration of the  $\text{KH}_2\text{PO}_4$ . Where  $\text{CaSO}_4$  and  $\text{CaCO}_3$  were employed they were added in the solid form when the solution was transferred to the culture vessels. Each culture vessel (1 pint jar of the Mason pattern) had a capacity of about 425 cc. and supported nine seedlings at the beginning of each culture series. The nutrient solutions were renewed after intervals of 3 days.

The plan of treatment of the solutions involved both the partial and complete replacement of  $\text{MgSO}_4$  with  $\text{Mg}(\text{NO}_3)_2$ . To the sulfur-free solution produced in the latter case, sulfur was also restored in the form of either  $\text{Na}_2\text{SO}_4$  or  $\text{CaSO}_4$ , each being made molecularly equivalent to the  $\text{MgSO}_4$  of the unmodified Knop solution. Still other portions of the sulfur-free solution were modified by adding either  $\text{NaNO}_3$  or  $\text{CaCO}_3$ , in twice molecular and molecular proportions respectively, as compared with the usual amount of  $\text{MgSO}_4$ . These last modified solutions served as controls for determining the effects of the sodium and extra calcium added elsewhere in the form of sulfates.

The method followed with the sand cultures was that of irrigation with a renewed nutrient solution, as previously employed by McCall.<sup>7</sup> Glazed, stoneware jars, about 16.0 cm. in diameter

<sup>5</sup> Knop, W., *Landw. Versuchsst.*, 1865, vii, 93.

<sup>6</sup> Tottingham, W. E., *Physiol. Researches*, 1913-17, i, 133. Shive, J. W., *ibid.*, 327.

<sup>7</sup> McCall, A. G., *Soil Science*, 1916, ii, 207.

and 11.0 cm. deep (2 quart jars), which conveniently held 3.5 kilos of fine sand, were employed as culture vessels. The inner surface of these jars was coated with paraffin, to prevent contamination of the nutrient solution by solutes from the stoneware. The sand employed consisted of spheroidal particles, most of which passed through a sieve with 70 meshes to the inch but were retained by one of 80 meshes. It was the product designated "banding sand" by the Ottawa Silica Company of Illinois. Although containing only traces of impurities, it was washed thoroughly with water before use. For the purposes of maintaining alkalinity and supplying iron, 3.0 gm. of  $\text{CaCO}_3$  and 1.0 gm. of  $\text{Fe}_2\text{O}_3$  were mixed with the portion of sand for each culture. Phosphorus was added to the nutrient solution in the form of  $\text{K}_2\text{HPO}_4$ . The application of nutrient solution was gradually increased from 25 to 100 cc. per culture at each renewal, and the period between renewals was gradually reduced from 4 to 2 days. As measured by Hilgard's method,<sup>8</sup> the water-holding capacity of the sand here employed was 23.4 per cent, by weight. By daily weighings, in connection with the use of a suction pump, the plane of total water content of all of the cultures was made uniform and was increased gradually from 20 to 22 per cent, by weight, of the dry sand. It was possible to measure approximately the loss of water through the plants, as the seedlings and irrigating funnel were sealed about with a mixture of vaseline and paraffin, but the data obtained showed no significant variations.

As none of the plants produced flowers in any of the culture series, it was difficult to select a stage of growth approximating maturity. Each series was harvested, however, when pronounced death of the basal leaves seemed to indicate a decided decline of growth with all of the cultures. After determining the average length of the three or four longest roots, and washing the latter parts, the plants were severed at the crown of the roots. The tops and roots were then dried at about  $100^\circ\text{C}$ ., for weighing. Except in the case of the sand cultures, the finely ground tops of the duplicate cultures were mixed for determining the average nitrogen content. In some cases, determinations of nitrogen in

<sup>8</sup> Hilgard, E. W., *Soils*, New York, 1906, 208-209.

the form of nitrates were also made. Total nitrogen was determined by the Gunning method<sup>9</sup> and nitrate nitrogen was determined by precipitation with nitron.<sup>10</sup> It seemed possible that the data derived from these analyses would indicate disturbances of protein synthesis, which might follow deficiencies in the sulfur supply of these clover plants.

Series 1 was conducted with water cultures. It covered a much longer period of growth than the succeeding series. After 37 days of growth the cultures were transferred to jars of a capacity of about 850 cc. (1 quart jars of the Mason pattern). The widely varying number of plants which survived in the different cultures tends to make the results of questionable value. However, the latter are unchanged in general trend if considered on the basis of unit plant per culture; and they are confirmed, in some respects, by the succeeding and more uniform series. The results appear in Table I.

Series 2, also conducted with water cultures, was disturbed by attacks from thrips. This may account partly for its comparatively early apparent maturing. Its more restricted growth period may, in turn, account for its less marked differences in yields, as compared with the other series. The results of this series appear in Table II.

Series 3, conducted with sand cultures, reached apparent maturity comparatively quickly. The seedlings were 10 days older than usual when transferred to the cultures, having been nourished for this period by irrigation with the sulfur-free modification of Knop's solution. Only six plants were started in each culture. The entire series was conducted upon a rotating table, after the manner of Shive<sup>11</sup>, to render uniform the effects of aerial environment with all of the cultures. The results appear in Table III.

A comparative survey of the results of these three series of cultures shows that 0.9 of the usual amount of  $MgSO_4$  in Knop's solution could be displaced, under the conditions of the water

<sup>9</sup> Official and provisional methods of analysis, *Assn. Official Agric. Chemists, U. S. Dept. Agric., Bureau of Chem., Bull. 107* (revised), 1907.

<sup>10</sup> Treadwell, F. P., *Analytical chemistry*, New York, 4th edition, 1915, ii, 451-453.

<sup>11</sup> Shive, *Physiol. Researches*, 1913-17, i, 344-345.

TABLE I.  
Yield and Nitrogen Content of Red Clover (*Trifolium pratense*) Grown 122 Days (Mar. 13—July 13, 1915) in Knop's Solution and Modifications Thereof.

Culture No.	Description of nutrient medium.	No. of plants measured.	Yield of dry tops.		Yield of dry roots.		Maximal length of roots.		Total nitrogen of tops.	
			Actual.	Relative.	Actual.	Relative.	Actual.	Relative.	Actual.	Relative.
			gm.	per cent.	gm.	per cent.	mm.	per cent.	gm.*	per cent.
1	Unmodified Knop solution.	3	3.252	—	0.924	—	119	—	—	—
2		3	3.585	100	1.008	100	103	100	3.47	0.119
3	0.9 MgSO <sub>4</sub> of Knop solution replaced by Mg(NO <sub>3</sub> ) <sub>2</sub> .	6	3.414	—	0.942	—	137	—	—	—
4		2	3.384	99	0.730	87	175	141	3.63	0.123
5	Total MgSO <sub>4</sub> of Knop solution replaced by Mg(NO <sub>3</sub> ) <sub>2</sub> .	6	2.220	—	1.248	—	250	—	—	—
6		8	1.952	61	1.304	132	225	214	4.46	0.093
7	Same as 5 and 6 + Na <sub>2</sub> SO <sub>4</sub> ≈ MgSO <sub>4</sub> of Knop solution.	4	3.388	—	1.044	—	131	—	—	—
8		3	3.075	95	1.113	112	119	113	3.65	0.118
9	Same as 5 and 6 + NaNO <sub>3</sub> ≈ MgSO <sub>4</sub> of Knop solution.	6	2.340	—	1.116	—	162	—	—	—
10		6	2.022	64	1.038	111	225	175	3.76	0.082
11	Same as 5 and 6 + CaSO <sub>4</sub> ≈ MgSO <sub>4</sub> of Knop solution.	4	4.392	—	0.876	—	144	—	—	—
12		6	4.386	128	1.194	107	137	127	3.84	0.109
13	Same as 5 and 6 + CaCO <sub>3</sub> ≈ MgSO <sub>4</sub> of Knop solution.	8	2.072	—	1.208	—	175	—	—	—
14		6	2.004	60	1.266	128	287	208	4.69	0.096
15	Same as 5 and 6 + CaSO <sub>4</sub> ≈ 3 × MgSO <sub>4</sub> of Knop solution.	4	4.352	—	1.209	—	100	—	—	—
16		2	3.708	118	0.904	109	175	124	3.63	0.146
17	Same as 5 and 6 + CaCO <sub>3</sub> ≈ 3 × MgSO <sub>4</sub> of Knop solution.	6	2.454	—	1.434	—	225	—	—	—
18		8	2.496	72	1.550	153	200	192	3.47	0.086

\* Average value of duplicate cultures.

TABLE II.  
Yield and Nitrogen Content of Red Clover Grown 64 Days (Apr. 13—June 16, 1916) in Knop's Solution and Modifications Thereof.

Culture No.	Description of nutrient medium.	No. of plants measured.	Yield of dry tops.		Yield of dry roots.		Maximal length of roots.		Total nitrogen of tops.	
			Actual.	Relative.	Actual.	Relative.	Actual.	Relative.	Actual.	Relative.
			gm.	per cent*	gm.	per cent*	mm.	per cent*	gm.†	per cent*
1	Unmodified Knop solution.	9	1.354	—	0.512	—	137	—	—	—
2		9	1.367	100	0.431	100	144	100	3.03	0.041
3	0.9 of $MgSO_4$ of Knop solution replaced by $Mg(NO_3)_2$ .	9	1.388	—	0.429	—	125	—	—	—
4		8	1.289†	98	0.471	95	106	82	3.40	0.046
5	Total $MgSO_4$ of Knop solution replaced by $Mg(NO_3)_2$ .	9	0.955	—	0.476	—	125	—	—	—
6		9	0.861	67	0.470	100	135	92	4.03	0.037
7	Same as 5 and 6 + $Na_2SO_4 \approx MgSO_4$ of Knop solution.	9	1.544	—	0.535	—	150	—	—	—
8		8	1.631†	117	0.572	117	150	106	3.20	0.051
9	Same as 5 and 6 + $NaNO_3 \approx MgSO_4$ of Knop solution.	8	1.158†	—	0.509	—	160	—	—	—
10		9	0.950	77	0.533	108	137	106	3.91	0.041
11	Same as 5 and 6 + $CaSO_4 \approx MgSO_4$ of Knop solution.	9	1.271	—	0.517	—	100	—	—	—
12		9	1.391	98	0.559	114	128	81	3.24	0.043
13	Same as 5 and 6 + $CaCO_3 \approx MgSO_4$ of Knop solution.	9	0.888	—	0.499	—	131	—	—	—
14		9	1.046	71	0.521	108	137	95	3.90	0.038

\* Average value of duplicate cultures.

† Converted to equivalent for nine plants.

TABLE III.  
Yield and Nitrogen Content of Red Clover Grown 54 Days (Jan. 7—Mar. 2, 1918) in Sand Irrigated with Knop's Solution and Modifications Thereof, with  $\text{CaCO}_3$  Added.

Culture No.	Description of nutrient medium.	No. of plants measured.	Yield of dry tops.		Yield of dry roots.		Maximal length of roots.		Total nitrogen of tops.		
			Actual.	Relative.	Actual.	Relative.	Actual.	Relative.	Actual.	percent†	Relative.
			gm.	percent*	gm.†	percent*	mm.	percent†	gm.*	percent*	percent
1	Unmodified Knop solution.	6	3.66	—	1.80	—	169	—	4.24	—	—
2		6	3.66	100	1.96	100	138	100	4.25	0.156	100
3	0.99 of $\text{MgSO}_4$ of Knop solution replaced by $\text{Mg}(\text{NO}_3)_2$ .	6	2.88	—	2.08	—	163	—	4.11	—	—
4		6	3.18	83	1.94	107	125	94	3.45	0.114	73
5	Total $\text{MgSO}_4$ of Knop solution replaced by $\text{Mg}(\text{NO}_3)_2$ .	6	1.50	—	0.48	—	150	—	3.43	—	—
6		6	2.22	51	1.12	43	200	114	4.31	0.074	47
7	Same as 5 and 6 + $\text{CaSO}_4 \approx \text{MgSO}_4$ of Knop solution.	6	5.04	—	2.76	—	169	—	4.04	—	—
8		6	4.92	136	3.08	155	169	110	4.00	0.201	129

\* Average value of duplicate cultures.

† Air-dried; containing sand.

cultures, without seriously disturbing the growth of the red clover plant. In the sand cultures, displacement of 0.99 of the usual amount of  $\text{MgSO}_4$  led to a considerably decreased production of dry matter of the stems and leaves. Displacement of the total  $\text{MgSO}_4$  led to decreases of 33 to 49 per cent in the production of dry tops in the various culture series. Addition of sulfur to the modified sulfur-free Knop's solution, in the forms of  $\text{Na}_2\text{SO}_4$  and  $\text{CaSO}_4$ , resulted in increased growth of the tops of the clover plant. In the majority of these cases the growth was decidedly superior to that with the unmodified Knop's solution. The results of the control cultures with  $\text{NaNO}_3$  and  $\text{CaCO}_3$  show that this effect is not attributable to the cations of these added sulfates, when supplied in certain other salts.

As regards the roots, the results are extremely variable. With the water cultures of Series 1 the effect of the various sulfates was to reduce the length of roots, as compared with the sulfur-free modification of Knop's solution, but this was not true of the other series of cultures.

The amount of nitrogen absorbed by these cultures fluctuated more or less parallel to the yields of dry tops, both of these values decreasing with the decrease of sulfur supply. It would seem logical to conclude that a deficiency of sulfur here operated to decrease the synthesis of protein and restrict the elaboration of plant tissue, with a concomitant decrease in the assimilation of nitrogen from the culture medium. That the percentage of assimilation of the absorbed nitrogen was not greatly different in the various cultures of each series here dealt with, is shown by the following percentages of the total nitrogen in the form of nitrate, as determined by analysis: Series 1, Cultures 1 and 2, 7.2 per cent; Cultures 3 and 4, 11.0 per cent. Series 3, Cultures 1 and 2, 7.5 per cent; Cultures 5 and 6; 6.2 per cent; Cultures 7 and 8, 7.5 per cent.

On the whole, the results of these cultures show a superiority of  $\text{Na}_2\text{SO}_4$  and  $\text{CaSO}_4$  over  $\text{MgSO}_4$ , as sources of sulfur for the red clover plant in water and sand cultures. This might be expected to follow the known toxic properties of magnesium salts. In harmony with the results previously reported from soil cultures<sup>2</sup>  $\text{CaSO}_4$  has proved an especially efficient source of sulfur.

In view of the fact that all of the nutrient media which here supplied sulfur were liberally supplied with calcium, it appears that  $\text{CaSO}_4$  had peculiar efficiency as a molecular complex.

#### SUMMARY.

The data presented here show that, for these experimental conditions, between 0.1 and 0.01 of the usual amount of  $\text{MgSO}_4$  of Knop's solution was as efficient as the usual amount for the growth of red clover, when the balance of the  $\text{MgSO}_4$  was replaced by  $\text{Mg}(\text{NO}_3)_2$ . The yield of dry tops of this plant was only one-half to two-thirds as great with  $\text{MgSO}_4$  wholly replaced by  $\text{Mg}(\text{NO}_3)_2$  as with the unmodified Knop's solution. Addition of  $\text{Na}_2\text{SO}_4$  and  $\text{CaSO}_4$  to the sulfur-free modification of Knop's solution, in amounts equivalent to the sulfur of the unmodified solution, produced yields of dry tops superior to those of the latter solution. In this respect,  $\text{CaSO}_4$  was very efficient. From the nature of the various nutrient media employed, it appears that the sulfur of this salt functioned in the molecular combination in which it was supplied. The more or less parallel fluctuations of plane of sulfur supply, weight of nitrogen assimilated, and yield of dry tops, of these clover plants, indicate that a deficiency of sulfur supply restricted growth by limiting the synthesis of protein.



## EXPLANATION OF PLATE 1.

FIG. 1. Series 1. Water cultures of *Trifolium pratense* after 99 days of growth. Plants 1 and 2, unmodified Knop's solution. Nos. 5 and 6, total  $\text{MgSO}_4$  of Knop's solution replaced by  $\text{Mg}(\text{NO}_3)_2$ . Plants 7 and 8, same as Nos. 5 and 6, plus  $\text{Na}_2\text{SO}_4 \approx \text{MgSO}_4$  of Knop's solution. Plants 11 and 12, same as Nos. 5 and 6, plus  $\text{CaSO}_4 \approx \text{MgSO}_4$  of Knop's solution. Plants 13 and 14, same as Nos. 5 and 6, plus  $\text{CaCO}_3 \approx \text{MgSO}_4$  of Knop's solution.

FIG. 2. Series 3. Sand cultures of *Trifolium pratense* after 53 days of growth. Plants 1 and 2, unmodified Knop's solution. Nos. 3 and 4, 0.99 of  $\text{MgSO}_4$  of Knop's solution replaced by  $\text{Mg}(\text{NO}_3)_2$ . Plants 5 and 6, total  $\text{MgSO}_4$  of Knop's solution replaced by  $\text{Mg}(\text{NO}_3)_2$ . Plants 7 and 8, same as Nos. 5 and 6, plus  $\text{CaSO}_4 \approx \text{MgSO}_4$  of Knop's solution.



FIG. 1.



FIG. 2.

(Tottenham: Red clover.



## STUDIES OF EXPERIMENTAL SCURVY.

### III. THE INFLUENCE OF MEAT AND VARIOUS SALTS UPON THE DEVELOPMENT OF SCURVY.\*

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(Received for publication, September 24, 1918.)

Gerstenberger<sup>1</sup> in a recent paper upon scurvy attributes the cause of the disease entirely to a deranged calcium metabolism. He states: "But it has seemed to me, in view of the known data regarding the pathology, chemistry and symptomatology of scurvy on the one hand, and regarding the important function played by calcium in bone growth, nerve conduction, vessel sealing, and cell permeability on the other, that all of these different system symptoms might be explained on the common basis of an interference with one or more of the normal functions of calcium and its physiological anion in the osseous system, vascular system, nervous system, muscular system and probably other systems." That calcium can play an important part in scurvy prevention is shown by the evidence presented in this paper, but that its metabolism alone is involved is questionable in the light of previous work from this laboratory.<sup>2,3</sup> Gerstenberger questions whether the guinea pigs that McCollum and I reported upon had only scurvy, and is of the opinion that they may also have had rickets. Our guinea pigs had the symptoms that in the literature are described as being characteristic of scurvy.

It is possible that the guinea pigs, or at least some of them may have had both scurvy and rickets. In my work I have found that guinea pigs kept on the same ration do not always show the same symptoms, but that they vary to a certain degree. This

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<sup>1</sup> Gerstenberger, H. J., *Am. J. Med. Sc.*, 1918, clv, 253.

<sup>2</sup> McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.

<sup>3</sup> Pitz, W., *J. Biol. Chem.*, 1918, xxxiii, 471.

has brought doubt in my mind as to whether or not what is commonly called scurvy in guinea pigs may not in reality be a complication of symptoms that overlap both scurvy and rickets, since both have a number of features in common.

Baumann and Howard<sup>4</sup> in a study of the mineral metabolism of normal and scorbutic guinea pigs found that all elements except magnesium and chlorine showed a negative balance during the scorbutic period. Bahrtdt and Edelstein<sup>5</sup> in a chemical analysis of bones from severe cases of scurvy found a distinct deficiency of calcium and phosphorus in the bones and muscles. Lust and Klocman<sup>6</sup> on the other hand, found that in a severe case of scurvy in an infant there was a retention of calcium, phosphorus, and chlorine, and especially of calcium during the active stage of the disease. During the convalescent stage of the case they found a negative mineral balance produced by the excessive excretion of salts and finally that in the period of healing the mineral balance was positive, except in the case of calcium, which was still negative. The differences in the findings of the above investigators is explained by Gerstenberger who showed that there is an increase in the calcification at Fränkel's line, indicating that scurvy is not a calcium deficiency disease; that while there is an excess of calcium deposited at that part of the bone at which endochondral growth takes place under normal conditions, there is nevertheless in all probability not so much calcium in the bone as a whole in advanced stages of scurvy as there would be in the normal bone; because, first, the bone has ceased to grow entirely or at any rate does not grow so rapidly as under normal conditions, and second, the normal process of resorption is going on, producing a rarefaction and brittleness with consequent loss of material.

Hess and Fish<sup>7</sup> have shown that the blood of infants suffering with scurvy shows a slight diminution in clotting power but this defect did not seem to be the result of an insufficiency of calcium. There was also an increased permeability of the vessel walls. Calcium is known to have a distinct function in controlling the

<sup>4</sup> Baumann, L., and Howard, C. P., *Am. J. Med. Sc.*, 1917, cliii, 650.

<sup>5</sup> Bahrtdt, H., and Edelstein, F., *Z. Kinderh.*, 1913, ix, 415.

<sup>6</sup> Lust, F., and Klocman, L., *Jahrb. Kinderh.*, 1912, lxxv, 663.

<sup>7</sup> Hess, A. F., and Fish, M., *Am. J. Dis. Child.*, 1914, viii, 385.

permeability of animal membranes,<sup>8-12</sup> and any interference with this function of calcium would increase the permeability of such membranes. Evidence of an increased permeability of the intestinal walls is shown by the fact that the blood serum of some guinea pigs suffering with scurvy gives positive precipitin tests to the proteins contained in the ration. This will be discussed in detail below.

A number of investigators have attempted to prevent the development of scurvy by the administration of phosphorated cod liver oil and tricalcium phosphate. Holst and Fröhlich<sup>13</sup> fed guinea pigs calcium in different forms without getting any beneficial results, and Gerstenberger<sup>1</sup> fed cod liver oil containing tricalcium phosphate, but without result. However, I have been able to protect guinea pigs against scurvy for 18 weeks by the ingestion of meat and tricalcium phosphate in a ration of rolled oats and milk. When tricalcium phosphate was fed in the above ration without the meat there was some beneficial effect noticed but it was not nearly so pronounced as when meat was introduced.

Hart, Miller, and McCollum<sup>14</sup> found that pigs fed a ration of corn, wheat middlings, oats, and oil meal, with a small quantity of roots and alfalfa hay included daily, grew at a slow rate for 4 to 5 months, after which growth ceased and the animals passed into a poor condition accompanied by lack of muscular coordination, emaciation, and labored breathing. The animals were unable to stand. When 1 per cent of commercial meat scraps was added to the above ration the animals grew at a normal rate, were vigorous, and in splendid condition, but the young were carried 1 or 2 weeks over time and were born dead. When 5 per cent of meat scraps was added the animals grew at a normal rate, were in excellent condition, and gave birth to fairly good litters. Some of the young, however, were weak and died. The

<sup>8</sup> Chiari, R., *Arch. exp. Path. u. Pharmacol.*, 1910, lxiii, 434.

<sup>9</sup> Chiari, R., and Januschke, H., *Arch. exp. Path. u. Pharmacol.*, 1911, lxv, 120.

<sup>10</sup> Overton, E., *Arch. ges. Physiol.*, 1904, cv, 176.

<sup>11</sup> Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 139.

<sup>12</sup> Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 423.

<sup>13</sup> Holst, A., and Fröhlich, T., *Z. Hyg. u. Infektionskrankh.*, 1912, lxxii, 1.

<sup>14</sup> Hart, E. B., Miller, W. S., and McCollum, E. V., *J. Biol. Chem.*, 1916, xxv, 239.

remarkable influence of the meat scraps is attributed by Professor Hart to an improvement of the proteins and to an appreciable increase in the calcium and phosphorus content of the ration through the introduction of bone material in the meat scraps. It was because of the splendid supplementary action of the meat scraps as shown by the work quoted above that the author investigated the effect of meat and tricalcium phosphate upon the development of scurvy.

The meat used in these experiments was lean beef that was obtained at a local butcher shop. The fat and connective tissue were cut away and the lean beef was put through the meat chopper. It was then dried in shallow granite pans over steam coils. After the meat was dry it was again ground in the meat chopper and stored in a tightly stoppered bottle. The rolled oats were ground up fine and the meat and salts mixed into it, so that the animals could not sort out any of the ingredients of the ration. All the animals in these experiments received fresh milk which was fed *ad libitum*.

Chart 1, Lot 80, shows the effect of the ingestion of 5 per cent of dried meat in the rolled oats and milk ration. All the animals on this ration developed scurvy during the 4th and 5th weeks except No. 5. This guinea pig died at the end of the 3rd week and showed lesions of scurvy. The other animals, although they developed scurvy during the 4th and 5th weeks, lived for a considerable time but did not gain in weight. Animals 7 and 8 were killed at the end of the 17th week. The meat apparently did not delay the onset of the disease to an appreciable degree since animals fed rolled oats and milk will develop scurvy during the 3rd and 4th weeks. *The meat did, however, greatly prolong the life of these animals, since guinea pigs fed on a diet of rolled oats and milk seldom live more than through the 5th week.* The meat may have supplemented the proteins of the oat and milk ration, or it may have acted as a stimulant to the appetites of the animals. One can hardly see how the meat served as an antiscorbutic in a capacity similar to phenolphthalein or mineral oil, as pointed out in a previous paper. One can, however, surmise how it may have supplemented a partially incomplete and already deficient intake of protein and thereby increased the constitutional resistance of the animal, and how it may also have stimulated appe-

tite with its resultant flow of digestive juices and concurrent proper alimentation. The element of increased resistance is not to be overlooked, especially when it is noted that while the time of onset of the disease was not delayed the period of life was prolonged considerably. It is certain that the protein intake on the basal ration of rolled oats and milk was far from satisfactory. In an experimental group of animals kept on sand to avoid consumption of litter a careful record of the proportionate amounts of food consumed indicated a protein intake equivalent to 10.2 per cent of the solids in the ration. Of this amount of protein 7.7 per cent was oat proteins and 2.5 per cent milk proteins. A comparison of the records of this group of animals with the records of animals given in Charts 3, 13, 14, and 15 demonstrates beyond a doubt that the meat acted primarily in improving and raising the level of the proteins of the ration. Since meat did not delay the onset of the symptoms of scurvy but did prolong the life of the animals it is highly probable that the inadequate nature or insufficient amount of proteins in the oat and milk ration may have been as much the cause of the death of the animals suffering from scurvy as was the disease. The food consumption of animals suffering with scurvy is very small during the active stages of the disease and the protein consumption is low at that time.

The guinea pigs in Chart 2, Lot 90, received a ration of rolled oats, meat, tricalcium phosphate, and milk during Period I. Three of these animals remained in excellent condition for 18 weeks and grew at a slow rate during that time. The animals were active, their food consumption was high, and they appeared normal in all respects. Animal 4 died at the end of the 4th week, but postmortem examination failed to show any of the characteristic lesions of scurvy. The tract of the animal was empty and it probably died of starvation. Animal 5, which was started the 13th week, showed slight symptoms of scurvy during its 5th week on the ration. It also had prolapse of the rectum. During the 18th week all animals lost weight and all but No. 1 developed scurvy. No. 2 died during the 19th week. In Period II 1 per cent of sodium chloride was added to the ration. The animals immediately improved in appearance, became more active, regained their appetites, and increased in weight, but the symptoms of scurvy remained. From the 18th to the 20th week the



food consumption of this group of animals had fallen off rapidly, but when NaCl was added to the ration their food consumption increased. 4 weeks after the introduction of NaCl into the ration the guinea pigs again began to lose weight and Animal 5 died 5 weeks later. It showed severe lesions of scurvy. Nos. 1 and 3 were killed at the end of the 30th week. Animal 1 did not show any lesions of scurvy but No. 3 did. The decided improvement of this ration over that of one of oats and milk is due in part to the 5 per cent of meat contained in the ration as shown by Chart 1, Lot 80, but whereas the animals in Lot 80 developed scurvy during the 4th and 5th weeks only two of the five animals in this group (No. 4 died at the end of the 5th week) developed scurvy before the 18th week. Clearly then the delay in the development of the disease was due to the 1 per cent of tricalcium phosphate in the ration. Calcium is known to function in maintaining normal permeability of animal tissues and it is in this capacity that the calcium probably acted. That there is an increase in the permeability of the intestinal and vessel walls of infants and guinea pigs suffering with scurvy is an established fact, and since calcium phosphate delayed the onset of the disease for a considerable time, the supposition seems justified that the calcium acted in maintaining normal permeability of these tissues and thus lessened the absorption of toxic products from the cecum and large intestine.

Calcium phosphate was found to be superior to other calcium salts, except perhaps calcium chloride, in delaying the onset of the symptoms of scurvy (see Charts 7, 8, 9, and 11). Since there is an abnormal accumulation of calcium at Fränkel's line, as shown by previously cited work, the calcium for this abnormal calcification may have been supplied by the calcium phosphate in the ration, because it is principally calcium phosphate that is concerned in bone formation; for this reason other calcium salts would not be as efficient. The case of calcium chloride will be discussed later in this paper.

During the disease the digestive tract is in an abnormal condition, the cecum and large intestine are greatly distended with putrefying feces, and normal peristalsis is interfered with. We may expect at the same time a decreased secretion of gastric juice and as a result a deficiency of HCl. The abnormal retention

of chlorine found to exist in an infant suffering with scurvy as reported by Lust and Klocman<sup>6</sup> indicates an abnormality in chlorine metabolism. That an actual deficiency of chlorine did exist in the ration fed these guinea pigs is shown by the fact that the ration contained only 0.075 per cent of chlorine. Dr. Babcock<sup>15</sup> showed that when milch cows were deprived of sodium chloride for long periods of time they soon reached a condition of low vitality in which a sudden and complete breakdown occurred. Recovery was rapid if NaCl was supplied. The condition was marked by loss of appetite, a lusterless eye, rough coat, and a very rapid decline in body weight.

The guinea pigs in this group, just previous to the addition of NaCl to the ration, behaved in a manner similar to that of the cows fed by Dr. Babcock; as in the case of the cows, recovery of the guinea pigs was rapid after NaCl was fed. This indicates that the manner in which the NaCl probably acted was in furnishing chlorine for greater HCl production in the gastric juice and in supplying a deficiency of this essential element with a concurrent increase in the constitutional resistance and improvement of the general well being of the guinea pigs.

The animals in Lot 91 (see Chart 3) received 10 per cent of meat in the ration. Animal 3 died during the 8th week and showed lesions of scurvy. The other animals did not show symptoms of scurvy until the 13th week. Animal 4 died at the end of the 16th week and No. 2 died at the end of the 24th week. Animal 1 was killed at the end of the 28th week and showed lesions of scurvy. When 5 per cent of meat was added to the rolled oats and milk ration the onset of the disease was not greatly delayed, but the life of the animals was prolonged. When 10 per cent of meat was added the onset of the disease was greatly delayed and the life of the animals was prolonged. This record is conclusive proof that the meat improved the ration through the introduction of higher levels and improvement of the proteins and by virtue of an increased intake of extractives and secretagogues which resulted in greater stimulation of appetite and its resultant increased flow of digestive juices. By improving the proteins and thus making the ration more nearly adequate for the physiological

<sup>15</sup> Babcock, S. M., *Wisconsin Agric. Exp. Station, 22nd Ann. Rep.*, 1905.

demands, and by stimulating the appetite and secretion of digestive juices, the resistance of the animals was increased and they were able to ward off the disease for a long time.

Chart 4, Lot 96, shows the result of feeding a ration of rolled oats, tricalcium phosphate, and milk. When the ration was not fortified with more protein, the tricalcium phosphate was unable to protect the animals to any marked degree. Animal 4 died at the end of the 2nd week and No. 5 at the end of the 6th week. Both of these animals died of scurvy. Nos. 2 and 3 showed symptoms of scurvy during the 5th week, but after the 6th week they increased in weight and probably would have lived for some time had they not been killed at the end of the 9th week for samples of blood. The tricalcium phosphate did delay the onset of scurvy to a very slight degree and prolonged the life of some of the animals. Apparently it is not so potent in delaying the onset of the disease as are lactose and laxatives,<sup>2</sup> and therefore unless the ration is improved as to its protein the tricalcium phosphate is not so effective as when protein is added to the ration.

In order to determine whether the calcium or the phosphoric acid radical or both were effective in causing the delay in the onset of the symptoms of scurvy other calcium salts and other salts of phosphoric acid were fed with the ration of rolled oats, meat, and milk. Chart 5, Lot 100, shows the effect of the addition of 1 per cent of dipotassium phosphate to the above ration. Animal 1 died at the end of the 3rd week and showed lesions of scurvy. No. 4 showed evidences of having the disease during the 4th week and died at the end of the 6th; No. 5 developed scurvy during the 10th week and died the 24th week; No. 6 never showed any signs of having scurvy. It died at the end of the 20th week, but no lesions of scurvy were observed. Since all the animals developed scurvy during the 4th week, except No. 5, which developed it during the 10th week, and No. 6, which never developed the disease, these animals did little better than those that received only the oats plus 5 per cent of meat. It is clear from this record that the dipotassium phosphate was of little benefit. Animal 6 apparently was an exceptional animal. Occasionally an animal will thrive on oats and milk. These results indicate that it is the calcium and not the phosphoric acid that is of prime importance in protecting the animal against scurvy.

To test further whether or not the calcium is instrumental in protecting the animals against scurvy, disodium phosphate was fed with the rolled oats, meat, and milk diet. Chart 6, Lot 103, gives the data of these experiments. The results in this case were no better than those obtained with the dipotassium phosphate. All the animals except No. 3 developed scurvy during the 4th week. No. 1 died at the end of the 5th week; No. 2 at the end of the 8th week, and No. 4 at the end of the 15th week. Animal 3 grew well and appeared to be normal, but when it was killed at the end of the 19th week a postmortem examination revealed a few lesions of scurvy. This record furnishes added proof that it is not the phosphoric acid, or at any rate not the phosphoric acid alone, that is instrumental in delaying the symptoms of scurvy.

The previous two records indicate that phosphoric acid alone played little or no part in protecting the guinea pigs against scurvy. Calcium alone or a combination of calcium and phosphorus must be necessary for protection against the disease. To analyze this point several other calcium salts were fed to guinea pigs with the rolled oats, meat, and milk ration. One group of animals (see Chart 7, Lot 104), was fed calcium acetate with the above ration. The amounts of calcium fed were the same in all these lots. The record of these animals indicates that although calcium is more effective in protecting the animals against scurvy than phosphoric acid, yet calcium acetate was not nearly so effective as was tricalcium phosphate. Since neither the calcium alone nor phosphoric acid was very effective in protecting these animals, the beneficial effects derived from tricalcium phosphate must have been due to that molecule rather than to any particular radical thereof. All the animals that received the calcium acetate developed scurvy about the 4th week. Animal 1 died at the end of the 4th week, No. 2 at the end of the 16th week, No. 3 at the end of the 12th week, and No. 5 at the end of the 5th week. Animal 4 grew at a rapid rate for 12 weeks and at no time did it show any symptoms of scurvy. It was killed at the end of the 20th week and on postmortem examination showed slight indications of scurvy.

Chart 8, Lot 105, shows the result of feeding a group of guinea pigs the above ration, but containing calcium carbonate in place

of calcium acetate. The results with these animals were much the same as those observed with the animals that received calcium acetate. All the animals developed scurvy during the 5th week and No. 1 died at the end of that week. Animal 3 died at the end of the 11th week. Up to the 10th week the other two animals grew a little, after which they did little better than maintain their weight.

The animals that received calcium lactate in place of calcium carbonate or acetate did a little better than did the animals on the previous two rations. Compare Chart 9 with Charts 7 and 8. Animal 1 died during the 3rd week, but failed to show any lesions of scurvy. The animal probably died of starvation. Only one animal, No. 2, showed symptoms of scurvy during the 4th week, but this animal lived for 20 weeks and grew rapidly throughout this period. When the animal was killed at the end of the 20th week it showed severe lesions of scurvy. No. 3 grew rapidly from the 10th week on, and never showed any evidence of having scurvy, nor could any lesions of scurvy be found when it was killed at the end of the 20th week. Animal 4 developed the disease during the 7th week and died at the end of the 9th week. Animals 5 and 6 developed scurvy during the 7th week and No. 6 died at the end of the 7th week, while No. 5 lived until the 16th week.

These results confirm the supposition that the beneficial effects of the tricalcium phosphate are due to the tricalcium phosphate molecule as a whole rather than to either the calcium or the phosphoric acid radical alone. Monobasic calcium phosphate was found to be less effective in delaying the development of scurvy than was tricalcium phosphate.

It will be recalled that the guinea pigs in Lot 90 (see Chart 2) began to lose weight, became inactive, and developed scurvy about the 18th week, and that when NaCl was added to the ration the animals improved within a week and gained in weight, but the symptoms of scurvy persisted. These animals already had severe scurvy, so that it is possible that the NaCl was added too late to be of any great aid to them. In order to determine whether or not guinea pigs would be entirely protected by the ingestion of NaCl in a ration of rolled oats, meat, tricalcium phosphate, and milk when fed to young animals at the beginning

of the feeding trial, a group of young guinea pigs was started on that ration (see Chart 10, Lot 117). All the animals suffered from diarrhea at the beginning of the experiment and two of them died. The other two animals recovered after the 6th week. Two additional animals were started on the ration 9 weeks later. One of these animals, No. 5, lost weight during the first 7 weeks, after which it gained in weight rapidly for a time and then decreased in its rate of growth. At no time, however, did this animal show symptoms of scurvy; No. 6 grew rapidly for 5 weeks and then declined in weight. All the animals in this group lost weight during the last 4 weeks of the feeding trial. The weather was very hot during this time, which was probably responsible for the decline in weight. However, only one animal, No. 4, showed positive symptoms of scurvy, while Animal 1 was questionable.

The records of this group of animals, as well as those of the animals given in Charts 11 and 12, are rather unsatisfactory due to the very hot weather prevailing during the experimental period. The animals were discarded sooner than they should have been because the author was called into military service. The results of the records of Chart 10, however, confirm those of Chart 2; namely, that  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  when fed with a ration of rolled oats, meat, and milk to guinea pigs greatly prolong the life and well being of the animals.

Since chlorine was found to be beneficial when fed with tricalcium phosphate in the oats and meat ration it was deemed advisable to feed calcium chloride with the oats, meat, and milk ration. Chart 11, Lot 118, is the record of these feeding trials.

After a slight decline in weight during the first 3 weeks of the feeding trial all the animals, except No. 4 which died at the end of the 2nd week, grew at a rapid rate. Animal 5 died at the end of the 6th week, but showed no lesions of scurvy. The remaining three animals grew very rapidly until the 10th week, after which time two of them declined in weight, but No. 2 continued to grow at a somewhat slower rate. Animals 1 and 3 showed slight symptoms of scurvy, but No. 2 never showed any signs of having the disease.

A comparison of the curves of these animals with those of the animals that received  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  shows that  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  are superior to  $\text{CaCl}_2$  in protecting guinea pigs against

scurvy when fed with rolled oats, meat, and milk. However, it must be remembered that the animals of Chart 2 were kept under more favorable conditions than were the animals of Chart 11. The animals of Chart 10, however, and those of Chart 11 were kept under similar conditions and the records of these two groups of animals can therefore be compared. A comparison of the records of these two groups of animals shows clearly that  $\text{CaCl}_2$  is somewhat superior to  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  in delaying the onset of scurvy in guinea pigs when fed with a ration of rolled oats, meat, and milk. These results demonstrate that it is calcium and chlorine that are mainly instrumental in delaying the onset of scurvy in guinea pigs and that the phosphorus plays a less important rôle in the development of this disease. They also show that calcium and chlorine when supplied in the same molecule as in  $\text{CaCl}_2$  are more effective in delaying the onset of the disease than when they are supplied in separate molecules as in  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$ .

In order to determine whether guinea pigs will do just as well when fed calcium chloride as when fed tricalcium phosphate and  $\text{NaCl}$  with the oats and milk ration, one lot of animals was fed calcium chloride with rolled oats and milk and another lot was fed tricalcium phosphate and  $\text{NaCl}$  with rolled oats and milk (see Chart 12, Lots 119 and 120).

A comparison of the two sets of records in Chart 12 shows that the  $\text{CaCl}_2$  when fed to guinea pigs with the rolled oats and milk ration is far superior to  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  in delaying the onset of scurvy. Those animals that received  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$ , Ration 119, did little better than animals kept on rolled oats and milk. Of course had these animals been kept under more favorable circumstances the records would have been better, but since both of the groups in Chart 12 were kept under the same conditions a comparison of the two sets of records is justifiable. All the animals of Ration 119, except No. 4, died before the end of the 5th week. No. 4 lived until the end of the 9th but showed no lesions of scurvy. The animals of Ration 120, that received  $\text{CaCl}_2$ , grew at a slow rate and were in much better condition than were the animals of Ration 119. Animal 1 died at the end of the 9th week, but showed no symptoms of scurvy; No. 3 was killed at the end of the 4th week and showed slight symptoms of scurvy.

Animals 2 and 5 lived for 14 weeks and grew at a slow rate throughout the feeding trial. No. 2 showed slight symptoms of scurvy the 9th week, but No. 5 never showed lesions of the disease. The animals were discarded at the end of the 15th week.

\* These records clearly demonstrate the superiority of  $\text{CaCl}_2$  over  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  in delaying the onset of scurvy in guinea pigs and illustrate how much more effective certain ions may be when combined in the same molecule than when present in different molecules.

Since  $\text{CaCl}_2$  was found to be superior to  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  in delaying the onset of scurvy in guinea pigs, calcium and chlorine must be of greater importance than phosphorus in the etiology of scurvy, and in this disease we must, therefore, have present a deficiency of chlorine as well as of calcium, or at least a decided derangement of chlorine metabolism. Such a deficiency or derangement of chlorine metabolism must result in a deficient production of  $\text{HCl}$  and a decrease in the digestive power of the gastric juice with a resultant improper functioning of the digestive tract.

To demonstrate further that the meat ingested with the ration of rolled oats and milk acted primarily in improving the proteins of the ration various other proteins were fed with rolled oats, milk, and tricalcium phosphate. Chart 13, Lot 101, is the record of a group of guinea pigs fed the above ration, plus 5 per cent of casein. That the casein did supplement the protein of the ration is evidenced by the results of this feeding trial (compare with Chart 4). Animal 5 developed scurvy the 3rd week and died at the end of that week; No. 4 developed the disease during the 9th week and died the 11th. None of the other animals showed any evidence of having scurvy. No. 2 died at the end of the 8th week, but no lesions of scurvy could be found, and No. 6 died at the end of the 7th week, but showed no evidences of having had the disease. Animals 1 and 3 grew at a rapid rate throughout the time of the experiment and appeared to be normal in all respects. They were killed at the end of the 24th week and showed no lesions of scurvy. The record of these animals is not so good as is that of animals fed meat in place of casein (see Lot 90, Chart 2). The meat therefore must have acted in a capacity other than simply in improving and increasing the protein content of the



ration to account for its superiority over the casein. What this was cannot be definitely pointed to at this time, but as stated before it probably stimulated the appetites of the animals and also stimulated secretions in the tract. Pawlow<sup>10</sup> showed that certain extractives found in meat stimulated a copious flow of gastric juice.

Chart 14, Lot 113, shows the result of feeding wheat gluten in place of casein in the above ration. As is to be expected the animals that received the wheat gluten did not do so well as those that received casein. Three of the animals developed scurvy the 5th week, while two of the animals did not develop the disease at all. The wheat gluten proved effective not in supplementing the other proteins of the ration, but simply in increasing the total protein content of the ration.

Liebig's extract was fed with the rolled oats, tricalcium phosphate, and milk ration to determine whether or not the meat had acted through its extractives as well as through the protein. Chart 15, Lot 114, gives the results of these experiments and shows that some beneficial effects were obtained by feeding the meat extract. The results were not nearly so good as those obtained with the meat or casein (see Charts 2 and 14). The animals all developed scurvy during the 4th and 5th weeks and all but one did very poorly throughout the feeding trial, but the life of these animals was prolonged. The protein contained in the meat extract was in part responsible for the beneficial effects noted, but the protein content of the meat extract was probably too low to account wholly for so great an effect as was noted.

#### *Precipitin Tests.*

In investigations on the effect of various rations on the growth of swine, Professor Hart has observed that this class of animals will pass into pathological conditions when confined to corn and milk. These animals suffer from severe constipation, dragging of the hind quarters, and swollen joints. He further showed<sup>16</sup> that the blood of these animals gave positive precipitin tests to corn and milk proteins, indicating an invasion of the blood stream through the intestinal walls of unhydrolyzed proteins of the ration.

<sup>10</sup> Unpublished data from this laboratory.

Since many of the symptoms that are shown by swine, kept on diets restricted to grains and small amounts of roughage or milk, are similar to some of the symptoms shown by guinea pigs suffering with scurvy, namely constipation, dragging of the hind legs, and swelling of the joints, precipitin tests were made upon the serums of scurvy guinea pigs. Fresh skimmed milk and oat protein were used for these tests and in some cases other proteins. To prepare the oat protein, finely ground rolled oats were stirred into hot, distilled water and allowed to cool. The mixture was then centrifuged and filtered through a Berkefeld filter. A clear, slightly yellowish solution was thus obtained. The milk and oat protein solutions were sterilized in an autoclave for 20 minutes at 15 pounds pressure. For the test with milk proteins 1 cc. of sterile milk was diluted with 50 cc. of sterile distilled water. All the tests were purely qualitative in nature, and no titrations were made to determine the amounts of precipitin contained in the blood serum. 1 cc. of the serum was diluted with 10 cc. of sterile, distilled water. The thoracic cavity of the animals was opened and the blood from the heart allowed to flow into a sterile Petri dish. After the blood had coagulated the clear serum was drawn off with a sterile pipette. For controls, the blood serum of normal guinea pigs kept on a diet of rolled oats and fresh grass was used.

In no case was a positive test observed with the serum of normal guinea pigs. The results of these tests are given in Table I. The corn and wheat proteins were prepared in the same manner as were the oat proteins. The egg protein was prepared by dissolving dried egg albumin in distilled water. All the protein solutions were sterilized.

A review of the data given in Table I shows that none of the animals that did not have scurvy gave positive precipitin tests with the proteins investigated, and that, while some of the animals that did have scurvy gave positive precipitin tests, others did not. All of the animals that had severe scurvy, except one, gave positive tests, although some gave strong, while others gave rather weak tests; in some cases the tests were so weak as to leave doubt as to whether the reaction was really positive. It is clear from this table that the stage of the disease apparently has no influence upon the response to the precipitin test. All that can be concluded thus far is that while normal guinea pigs do not

give a positive precipitin test to oat and milk proteins some scurvy animals do, but that the test is no indication of the severity of the disease. The precipitin test for oat and milk proteins was not specific, but also was given by corn and wheat protein. It has been found by several investigators that precipitins are

TABLE I.

*Results of the Application of Precipitin Tests to the Serum of Normal and Scurvy Guinea Pigs.*

Ration.	Stage of disease.	Protein.				
		Oat.	Corn.	Wheat.	Milk.	Egg.
1 Oats and grass.....	Normal.	0	—	—	0	—
2 " " ".....	"	0	—	—	0	—
3 " " ".....	"	0	—	—	0	—
4 " " ".....	"	0	—	—	0	—
5 " " milk.....	Severe scurvy.	++	—	—	++	—
6 " " ".....	"	++	—	—	++	—
7 " milk, and $\text{CaCl}_2$ .....	Slight	+	—	—	+	—
8 " and milk.....	Severe	+	—	—	+	—
9 " meat, milk, and $\text{CaCO}_3$ .....	"	?	—	—	?	—
10 " and milk.....	Slight	0	—	—	0	—
11 " meat, and milk.....	"	++	+	+	+	0
12 " " " ".....	"	++	+	+	+	0
13 " Liebig's extract, $\text{Ca}_3(\text{PO}_4)_2$ , milk.....	Severe	0	—	—	0	0
14 " heated meat, $\text{Ca}_3(\text{PO}_4)_2$ , milk.	"	++	+	+	+	0
15 " wheat gluten, $\text{Ca}_3(\text{PO}_4)_2$ , milk	"	?	—	—	?	—
16 " casein, $\text{Ca}_3(\text{PO}_4)_2$ , milk.....	No	0	—	—	0	—
17 " meat, $\text{Na}_2\text{HPO}_4$ , milk.....	Slight	+	—	—	+	—

0 = no tests given

++ = strongly positive.

+ = positive.

? = so slight as to be questionable.

— = no tests made.

not always specific.<sup>17</sup> The data here reported are still incomplete and are given merely as a preliminary report. The problem is being investigated further in this laboratory.

<sup>17</sup> Longcope, W. T., *Harvey Lectures*, 1915-16, xi, 271.

The presence of certain foreign bodies that give positive precipitin tests to oat, corn, wheat, and milk proteins in the blood stream of some guinea pigs suffering with scurvy gives rise to the question as to whether their presence in the blood is due to an increased permeability of the tissues, or to lesions in the intestinal walls. From the accumulation of evidence that in scurvy there is an increase in the permeability of the tissues, and in view of the fact that an ingestion of calcium phosphate or calcium chloride into the diet delayed the development of the disease, it is highly probable that the presence of foreign proteins in the blood stream is probably due to the increased permeability of the tissues. This does not exclude the possibility of lesions in the intestines playing a part in allowing protein molecules to pass into the blood stream. In order to determine whether lesions do exist in the intestinal walls of guinea pigs suffering with scurvy an examination of the intestinal walls of scurvy animals is being made in this laboratory.

#### SUMMARY.

The evidence given in the three papers upon scurvy from this laboratory shows that while the physical character of the diet is of great importance in preventing scurvy other factors enter into the causes of the disease.

In this paper it is shown that an improvement of the protein of the diet will protect guinea pigs from scurvy for a number of weeks and will greatly prolong the life of the animals, even though the physical character of the diet has not been improved. But while laxatives<sup>2</sup> and lactose<sup>3</sup> will prevent the development of scurvy in guinea pigs for 20 weeks, improvement in the protein of the diet, even when 10 per cent of meat is added, will not protect them for more than 13 weeks. When tricalcium phosphate was added with the meat the animals were protected for 18 weeks. Sodium chloride when added to the milk, rolled oats, meat, tricalcium phosphate ration, also effected some further protection, demonstrating that low chlorine is one among the many factors that may cause delay in the development of scurvy. This latter point is further emphasized by the remarkable protection afforded guinea pigs against scurvy by the ingestion of  $\text{CaCl}_2$  in the ration and shows that the calcium and chlorine ions are of greater importance in the development of this disease than is phosphorus.

The physical character of the diet and the character of the flora of the digestive tract are clearly of prime importance in the production of this disease, but other factors, such as those which make the diet more nearly chemically complete, which stimulate appetite and increase the flow of digestive juices and increase the resistance of the animals, which decrease the permeability of the intestinal wall, and which aid in correcting a deranged chlorine metabolism, are of great importance and will protect the animals from scurvy for a considerable time. *These experiments point to the little emphasized rôle of calcium salts in nutrition; namely, that of controlling the permeability of various animal tissues and thereby affording protection against invading agents.*

The sequence of events in the development of scurvy may be pictured thus: As feces accumulate in the cecum and large intestine of the animal and constipation has set in, peristalsis is decreased and the intestinal juices are secreted in less profusion, resulting in a lowering of the digestive power of the intestine and stomach and probably in the production of lesions in the intestines. Then, due to an increased permeability of the vessel walls, bacteria may invade the joints, as shown by Jackson and Moody,<sup>18</sup> toxic products are absorbed in greater amounts from the cecum and large intestine, and in some cases the whole protein molecule passes through the walls of the capillaries of the intestines into the blood stream.

No claim is made that the retention of feces is the primary factor in the production of scurvy, but it is clearly a contributing and probably a very important factor in the development of the syndrome of this disease.

<sup>18</sup> Jackson, L., and Moody, A. M., *J. Infect. Dis.*, 1916, xix, 511.

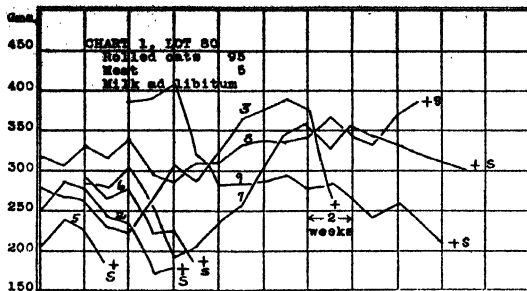


CHART 1. Lot 80 shows the effect of adding 5 per cent of meat to a ration of rolled oats and milk fed *ad libitum*. Animal 5 died at the end of the 3rd week and showed lesions of scurvy. All the other animals developed scurvy during the 4th and 5th weeks. In spite of this, Animals 3, 7, 8, and 9 lived for a considerable time. Animals 7 and 8 were killed at the end of the 17th week. The meat did not delay the onset of the disease but it did prolong the lives of a number of the animals for a long time.

S+ on the charts denotes that the animals had scurvy at the time of death; + signifies death without scurvy.

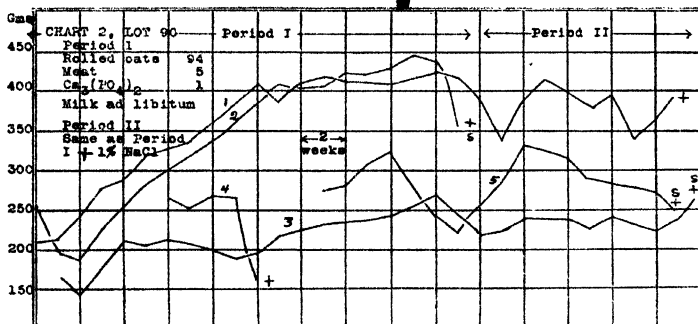


CHART 2. Lot 90. The animals in this lot received in addition to the ration of rolled oats, meat, and milk fed *ad libitum* (Ration 80), 1 per cent of  $\text{Ca}_3(\text{PO}_4)_2$  during the first period. Animal 4 died at the end of the 4th week, but it did not show any lesions of scurvy. No. 5 developed scurvy during the 5th week. The other three animals remained in excellent condition for 18 weeks when they began to lose weight and showed evidences of having scurvy. No. 1, however, although it lost in weight did not develop scurvy. No. 2 died at the end of the 19th week. In Period II, 1 per cent of NaCl was added to the ration. The animals all improved in appearance, became more active, and gained in weight for a time, but the symptoms of scurvy did not disappear. 4 weeks after the ingestion of NaCl into the ration they again lost in weight and Animal 5 died 5 weeks later. Nos. 3 and 1 were killed at the end of the 30th week. No. 3 showed lesions of scurvy while No. 1 did not.

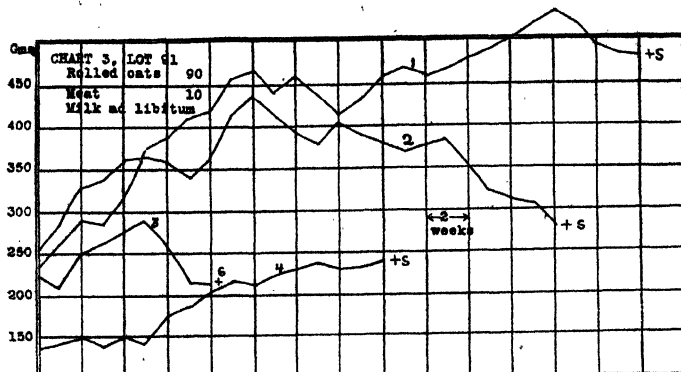


CHART 3. Lot 91 illustrates the behavior of guinea pigs receiving a ration of rolled oats and 10 per cent of meat and milk fed *ad libitum*. Animal 3 developed scurvy during the 5th week and died the 8th week. No. 4 died the 16th week, and No. 2 the 24th week. Animal 1 was killed the 28th week and showed lesions of scurvy. The record of these animals is considerably better than that of the animals that received 5 per cent of meat (see Chart 1, Lot 80), and shows that insufficient protein is ingested in the rolled oats and milk ration.

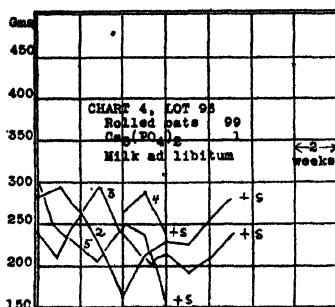


CHART 4. Lot 96 shows the results obtained by feeding  $\text{Ca}_3(\text{PO}_4)_2$  without meat in the rolled oats and milk ration. The  $\text{Ca}_3(\text{PO}_4)_2$  alone was unable to protect the animals against scurvy or to prolong their lives for any appreciable time; but when meat was added to this ration it was very efficient in protecting the animals against scurvy and in prolonging their lives (see Chart 2, Lot 90). Animal 4 died of scurvy at the end of the 2nd week. The other animals all developed scurvy during the 4th and 5th weeks, and No. 5 died at the end of the 6th week. Animals 2 and 3 increased in weight after the 6th week, but were killed at the end of the 9th week.

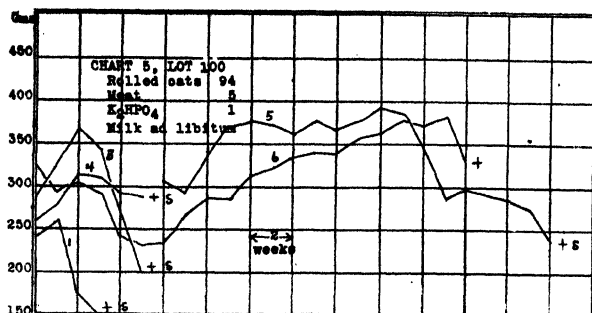


CHART 5. Lot 100 shows the effect of the addition of  $K_2HPO_4$  to the rolled oats, meat, and milk ration. These guinea pigs did little better than did guinea pigs that received only rolled oats, meat, and milk. Animals 1, 3, and 4 developed scurvy during the 3rd week; No. 1 died at the end of the 3rd week, and Nos. 3 and 4 at the end of the 5th week. No. 5 developed scurvy during the 10th week and died the 18th week; No. 6 never developed scurvy. It died at the end of the 19th week, but showed no lesions of scurvy.

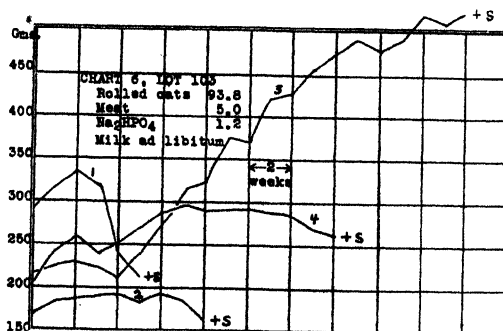


CHART 6. Lot 103. The animals in this group were fed the same ration as those in Lot 100 but in place of  $K_2HPO_4$  they were given  $Na_2HPO_4$  in the ration. These animals did no better than did those that received  $K_2HPO_4$  in this ration (see Chart 5). All the animals except No. 3 developed scurvy during the 4th week. No. 1 died at the end of the 5th week, No. 2 during the 8th week, and No. 4 at the end of the 14th week. Animal 3 grew rapidly and appeared to be normal, but when it was killed it showed a few slight lesions of scurvy.



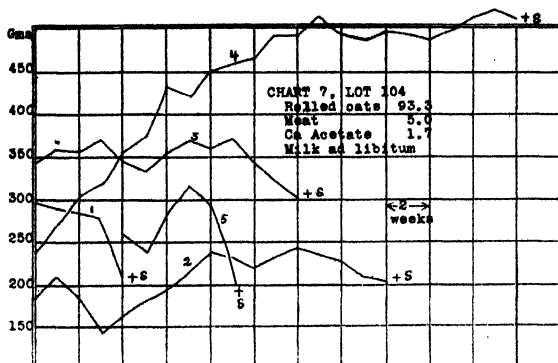


CHART 7. Lot 104 illustrates the behavior of guinea pigs fed the rolled oats, meat 5, and milk ration plus calcium acetate. The amount of calcium in this ration is the same as that fed as  $\text{Ca}_3(\text{PO}_4)_2$  (see Chart 2, Lot 90). All the animals in this group developed scurvy during the 4th week, and No. 1 died at the end of that week; No. 2 died at the end of the 16th week; No. 3 at the end of the 12th week, and No. 5 at the end of the 5th week. Animal 4 grew rapidly for 12 weeks and then maintained its weight, and at no time did it show evidences of having scurvy; but when it was killed at the end of the 20th week it showed a few slight lesions of scurvy. Although the calcium acetate did improve the rolled oats, meat, and milk ration it did not prove as effective as was  $\text{Ca}_3(\text{PO}_4)_2$  (see Chart 2, Lot 90).

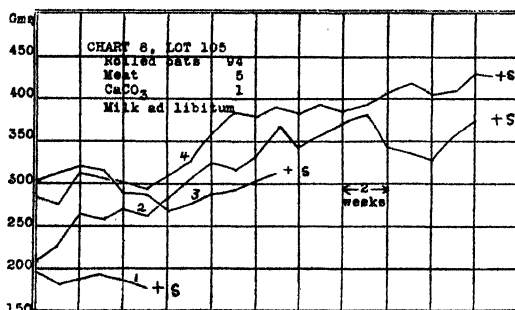


CHART 8. Lot 105 shows that calcium carbonate was no more efficient than calcium acetate in the nutrition of the guinea pig. All the animals developed scurvy during the 5th week and No. 1 died at the end of that week. No. 3 died at the end of the 11th week; No. 2 was killed at the end of the 20th week at which time it had severe scurvy; No. 4 was killed at the end of the 21st week and showed only slight lesions of scurvy. This animal had apparently recovered from the disease to a marked degree.

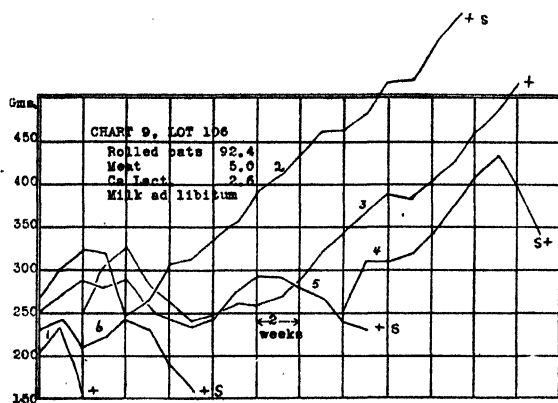


CHART 9. Lot 106 received calcium lactate in place of the carbonate. The curves show that the animals did somewhat better than did those that received calcium acetate (see Chart 7, Lot 104) or those that received calcium carbonate (see Chart 8, Lot 105). Animal 1 died after the 2nd week, but failed to show lesions of scurvy. Animal 2 showed symptoms of scurvy during the 4th week, but it gained in weight rapidly until it was killed at the end of the 20th week and showed severe lesions of scurvy. No. 3 never developed scurvy and grew rapidly. It showed no lesions of the disease when it was killed at the end of the 20th week. No. 4 developed scurvy during the 7th week and died the 9th week. Animals 5 and 6 developed scurvy during the 7th week and No. 6 died at the end of this week, while No. 5 lived until the 16th week.

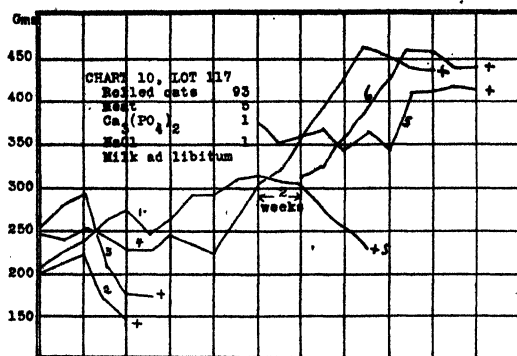


CHART 10. Lot 117. This chart is the record of a group of guinea pigs kept on Ration 80 plus  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$ . During the first 4 weeks all the animals except Nos. 4, 5, and 6 developed scurvy. Animal 2 died at the end of the 4th week and No. 3 at the end of the 5th week. Neither of these animals showed lesions of scurvy. Animal 1 died at the end of the 15th week. It was questionable whether or not this animal had scurvy. Animal 4 was discarded after the 18th week, and although it lost weight the last 4 weeks, due apparently to the hot weather, it did not show symptoms of scurvy. Animals 5 and 6 were started on the ration 9 weeks later than the other animals. Animal 5 did not begin to gain in weight until the 7th week after which it gained in weight until the 11th week. The animal, however, developed scurvy during the 9th week. No. 6 grew rapidly until the 5th week when it slowly declined in weight. It was discarded the 8th week and was in good condition at that time.

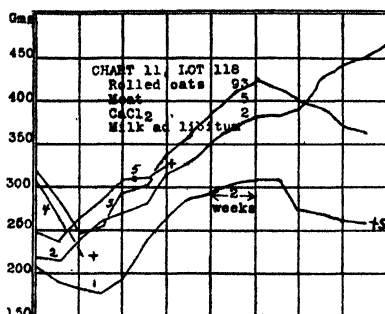


CHART 11. Lot 118 shows the effect of the ingestion of  $\text{CaCl}_2$  into Ration 80. Animal 4 died at the end of the 3rd week, but showed no signs of scurvy. No. 5 died during the 7th week. The intestine and stomach were greatly distended with gas, but the animal showed no lesions of scurvy. Animals 1 and 5 grew rapidly in weight until the 10th week after which time they slowly declined. They were discarded the 15th week and both showed symptoms of scurvy. Animal 2 grew rapidly throughout the feeding trial and when discarded, the 16th week, it was in good condition. The animals in this group did considerably better than did those that received  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  (see Chart 10).

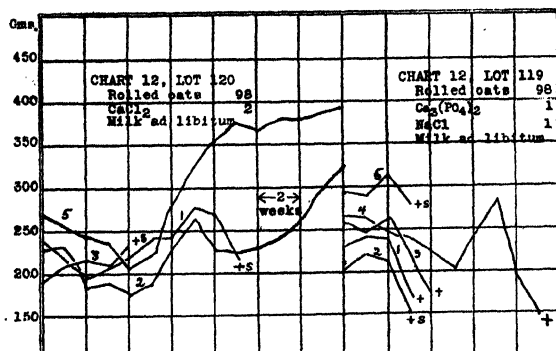


CHART 12. Lot 120 illustrates the behavior of guinea pigs fed upon a diet of rolled oats,  $\text{CaCl}_2$ , and milk fed *ad libitum*. Animal 3 died at the end of the 4th week and showed lesions of scurvy. Animal 1 grew a little but died at the end of the 9th week and showed decided lesions of scurvy. Animal 2 grew at a slow rate and showed symptoms of scurvy the 9th week. Animals 2 and 5 were discarded the 15th week; No. 5 grew until the 9th week after which it maintained its weight, but at no time did it show evidences of having scurvy. These animals did considerably better than those that received  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{NaCl}$  (see Lot 119, this chart).

Lot 119 was fed rolled oats,  $\text{Ca}_2(\text{PO}_4)_2$ , NaCl, and milk fed *ad libitum*. The animals all lost in weight after the 2nd week, but only one, No. 2, showed symptoms of scurvy. No. 1 died the 4th week, but showed no lesions of scurvy. No. 2 died the same week and did show lesions of scurvy. No. 3 died at the beginning of the 5th week, but showed no lesions of scurvy, while No. 5 died the 4th week and did show lesions of scurvy.

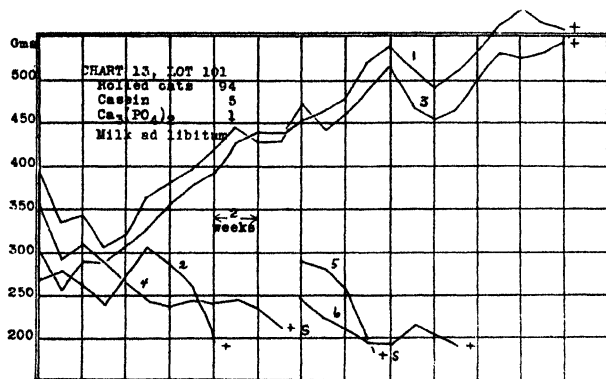


CHART 13. Lot 101 shows the effect of adding casein to a ration of rolled oats,  $\text{Ca}_3(\text{PO}_4)_2$ , and milk fed *ad libitum*. These guinea pigs did not do so well as those that received meat in place of casein (see Chart 2, Lot 90), but the casein did supplement the proteins of the ration (compare Chart 4). Animal 5 developed scurvy during the 3rd week and died at the end of that week. No. 4 developed the disease the 9th week and died the 11th week. None of the other animals showed evidence of having scurvy. Animals 1 and 3 were killed at the end of the 24th week and showed no lesions of scurvy.



# A METHOD FOR THE IDENTIFICATION OF CERTAIN URAMINO-ACIDS IN THE PRESENCE OF AMINO-ACIDS AND OF UREA.

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(Received for publication, September 25, 1918.)

## INTRODUCTION.

The facility with which uramino-acids or their anhydrides, hydantoins, may be formed from amino-acid and urea *in vitro* is shown by the work of Baumann and Hoppe-Seyler (1), Lippich (2), as well as by the critical studies of Salkowski (3), Dakin (4), Weiland (5), and most recently Abel, Rowntree, and Turner (6). Where uramino-acids or hydantoins have been found in biological fluids, urea has been present (7), amino-acids were not excluded, and in some work amino-acids were added with a view to further the synthesis *in vivo* of uramino-acids (8). Due to the fact that certain uramino-acids are compounds difficultly soluble in water and are extracted by ether (9) as well as by acetic-ether (4), they have been obtained as crystalline compounds in procedures for the isolation of other substances such as amino-acids (8, 9, 6), from biological fluids, and have occasionally served as evidence for the presence of amino-acids when these acids could not themselves be isolated.

As the question of the existence of preformed uramino-acids in body fluids has been left open (5, 6), it seemed advisable to undertake to modify the procedure hitherto used for the isolation of uramino-acids by taking advantage of the action of urease (10) for the decomposition of urea and thus secure its elimination from the material to be investigated. It is possible, also, to follow more closely traces of uramino-acids by an application of the Van Slyke gasometric method for the determination of amino nitrogen (11). The uramino-acids studied give off amino nitro-



gen in the course of an hour as contrasted with amino-acids which give it off quantitatively in 4 minutes, with the exception of lysine which takes longer. The anhydrides of the uramino-acids, as was to be expected, give off no amino nitrogen. Therefore by forming anhydrides from uramino-acids, amino nitrogen suspected to be lysine for example, may be identified as having its source from uramino-acid or from amino-acid according as to whether it disappears or remains after the procedure of anhydride formation.

The chief evidence for the presence of preformed uramino-acid is an experiment carried out by Dakin (12) where ureidophenylpropionic acid crystallized from cat's urine without analytical procedure. In this experiment doses of 6 to 8 gm. of phenylalanine were administered intravenously to cats during  $1\frac{1}{2}$  hour periods and the urine was collected during the ensuing 11 hours. The urine on standing contained at the edge of the vessel crystals which were identified as ureidophenylpropionic acid. Larger quantities of the uramino-acid were obtained by acidifying the urine with phosphoric acid, extracting in a continuous extractor, distilling the acetic-ether with steam, clarifying with animal charcoal, concentrating the aqueous residue, and allowing to crystallize.

Abel, Rowntree, and Turner (6) in referring to isobutylhydantoin, isolated by them from a dialysate from circulating dog's blood, say: "The isolation of this substance does not necessarily prove its original presence in the blood of the dog, inasmuch as it may have been formed during the ester distillation by a condensation of urea with another substance." The possibility that this other substance may be leucine is pointed out. The question "whether this and perhaps other hydantoin derivatives occur in the animal body" (6) made it desirable to make the following experiments.

A study of  $\alpha$ -ureido- $\beta$ -phenylpropionic acid and of  $\alpha$ -ureido-isobutylacetic acid has been carried out. In applying the procedures here described to the question of preformed  $\alpha$ -ureido- $\beta$ -phenylpropionic acid, it was thought advisable to conform as nearly as possible to the conditions under which Dakin isolated 1.5 gm. of this uramino-acid. The same choice of animal, of dosage of phenylalanine, of time of injection of amino-acid, and

of collection of urine was made, and Dakin's method of extraction used.

*Method.*

The fresh material is treated with Jack bean urease in such proportion as to decompose the urea in 15 minutes (10), at 37°C., or when possible the collections are made into enzyme solutions at this temperature. After testing a portion of the material to make certain all urea is decomposed, the whole amount is neutralized with phosphoric acid and an excess added. The acid fluid is then extracted in a liquid extraction apparatus for 6 or more hours with four to six volumes of acetic-ether. The acetic-ether is then distilled with steam, the aqueous residue clarified with charcoal, and concentrated on the water bath to small volume. Crystals of uramino-acid, if present, are filtered off, dried, and identified by means of the melting point and of amino nitrogen determinations before and after treatment with boiling hydrochloric acid. If no crystals separate and traces of uramino-acids are to be excluded, the concentration may be continued until a small amount of syrup results which is then taken up in 1 to 2 cc. of water, normal sodium hydroxide added drop by drop until alkaline, and filtered if necessary. The material is then boiled for 1 minute to drive off traces of ammonia and made up to volume in a 10 cc. flask. Determinations on the amino nitrogen content may then be made. 2 cc. samples are taken and the determination is carried out as for amino-acid. Second samples of material are shaken 1 minute and then allowed to stand 1 hour in the reaction chamber, and shaken a second time for 3 minutes. If in the second procedure more amino nitrogen is found than in the first a 5 cc. portion of the material is acidified with ten drops of concentrated hydrochloric acid and boiled under a reflux condenser for an hour. The material is made alkaline and again made up to volume and determinations on the amino nitrogen are run. The difference between the amino nitrogen figure before boiling with hydrochloric acid and that after boiling may be assumed to be amino nitrogen from uramino-acid.

EXPERIMENTAL.

A cat weighing 2.35 kilos was given by stomach tube 4.7 gm. of urethane dissolved in warm saline solution. The left femoral

vein was cannulated and connected to a burette containing 300 cc. of normal salt solution in which 6.0 gm. of *dl*-phenylalanine were dissolved. The bladder was exposed, the urethra tied close to the bladder and the latter punctured, the urine withdrawn, and a glass bladder cannula inserted and tied in place. The bladder was replaced in the peritoneal cavity, and the incision closed by clamps. The cannula was so bent that the urine dropped into a 50 cc. beaker which stood in a bath of water at 37–45°C.

The phenylalanine and salt solution were slowly and continuously run into the vein for 1½ hours.

5 cc. of Jack bean extract (10) were placed in the beaker to receive the urine as excreted. This extract was constantly agitated, and the temperature kept between 37–40°C. When 5 cc. of urine had been collected, a fresh specimen of extract was taken for the next collection.

The alkalinity of a 2 cc. portion of the Jack bean extract and urine was determined, and after a 15 minute interval with the material at 37°C., a second 2 cc. portion was titrated, and the completion of the decomposition of urea was verified. Portions of 60 cc. of urea-free material were neutralized with phosphoric acid and 2 to 3 cc. of acid were added in excess and introduced into a continuous extraction apparatus. Approximately 370 cc. of ethyl acetate were in circulation in the extraction apparatus. Extraction was carried on for 6 hours on each specimen, the acetic-ether in the distilling flask (75 cc.) was then removed, and distilled with steam, while the next specimen of acidified material was extracted with the addition of 75 cc. of fresh acetic-ether.

The slightly colored aqueous residues left on distillation with steam were clarified with animal charcoal, filtered, evaporated to 1 to 2 cc. on the water bath, and allowed to stand for 24 hours. No crystallization of uramino-acids took place.

The drops of brown syrup-like material were then examined under the microscope for crystals. Only a trace of amorphous substance was present.<sup>1</sup> The material was taken up in 2 cc. of

<sup>1</sup> Two preliminary experiments, carried out in the same manner as the experiment here reported, gave similar residues with no crystallization of uramino-acid, but the exclusion of uncrystallized uramino-acid in the syrup by the application of the amino nitrogen determination was not done.

normal sodium hydroxide, 5 cc. of water were added, the material was boiled to drive off traces of ammonia present, and then made up to 10 cc. in a measuring flask. Portions of 2 cc. each were analyzed for amino nitrogen.

A bare trace of amino nitrogen was obtained in each residue in the 4 minute determination which was not increased by an hour's reaction with nitrous acid. A control extraction of 50 cc. of normal cat's urine and 50 cc. of Jack bean extract gave a small correction which covered the trace of amino nitrogen found.

Amino nitrogen determinations upon ureidophenylpropionic acid upon its anhydride, benzylhydantoin, upon ureidoisobutylacetic acid and its anhydride, isobutylhydantoin, gave the following figures:

Amount.	Substance.	NH <sub>2</sub> -N			
		Time of reaction.			Theory for 50 per cent N.
		4 min.	30 min.	1 hr.	
mg.		mg.	mg.	mg.	mg.
16.0	Ureidophenylpropionic acid.	0.26	1.03	1.34	1.07
20.2	Benzylhydantoin.			0	0
10.8	Ureidoisobutylacetic acid.	0.20	0.87	0.94	0.84
23.3	Isobutylhydantoin.			0	0

These uramino-acids were made from amino-acids yielding the theoretical amounts of amino nitrogen. A nitrogen determination on each would indicate their purity; however, slightly more than 50 per cent of the nitrogen of these compounds was usually obtained after 1 hour's interaction with nitrous acid. After 1 hour no more nitrogen was obtained.

Amount.	Substance.	Total nitrogen <sup>a</sup>	
		Found.	Theory.
mg.		mg.	mg.
89.4	Ureidophenylpropionic acid.	14.46	14.38
160.4	Ureidoisobutylacetic acid.	22.13	21.82

The specificity of the action of urease has been emphasized by Marshall and figures given to indicate no action upon  $\alpha$ -ureido-

$\beta$ -phenylpropionic acid (10). The following determination shows no action upon  $\alpha$ -ureidoisobutylacetic acid. 0.05 gm. of  $\alpha$ -ureidoisobutylacetic acid was dissolved in a few drops of normal sodium hydroxide and made up to a volume of 10 cc. with water. 10 cc. of Jack bean extract were added. 4 minute determinations of amino nitrogen were made at once and after 15 hours.

Sample.	Uramino-acid.	Time.	NH <sub>2</sub> -N
cc.	mg.	hrs.	mg.
2	5	0	0.33
2	5	15	0.33

*Recovery of Added  $\alpha$ -Ureido- $\beta$ -Phenylpropionic Acid.*

To 10 cc. of cat's urine and 10 cc. of Jack bean extract which had stood at room temperature 12 hours and was urea-free, were added 0.2 gm. of  $\alpha$ -ureido- $\beta$ -phenylpropionic acid and 1 cc. phosphoric acid, and an extraction experiment was carried out as above.

Crystals separated in the aqueous residue on concentration and after standing 12 hours were filtered from 4 cc. of mother liquor. 0.15 gm. of slightly colored crystals, having the same melting point as the uramino-acid added (185° uncorrected), were recovered. Mixing the synthetic and the product recovered from the urine gave no depression in the melting point. On recrystallizing a part of the product the melting point was not raised. The material was extracted for a further 6 hours, and the acetic-ether in the distilling flask again treated as before. No crystals were obtained from the aqueous residue.

I. Mother liquor from crystalline uramino-acid recovered.

NH<sub>2</sub>-N determined in 30 minutes reaction with HNO<sub>2</sub>... 1.5 mg.

Uramino-acid equivalent to this N..... 22.0 "

Calculated on a basis of NH<sub>2</sub>-N. 50 per cent of total N of compound.

II. Residue from second extraction of original material.

NH<sub>2</sub>-N..... 1.0 mg.

Uramino-acid equivalent..... 14.0 "

Total uramino-acid recovered..... 0.186 gm.

0.095 gm. uramino-acid dissolved in normal sodium hydroxide and made up to 10 cc. with water. 2 cc. specimen analyzed.

NH <sub>2</sub> -N after $\frac{1}{2}$ hr.....	1.3 mg.
0.095 gm. uramino-acid recovered from extraction experiment dissolved in normal sodium hydroxide and made up to 10 cc. 2 cc. specimen analyzed.	
NH <sub>2</sub> -N after $\frac{1}{2}$ hr.....	1.3 mg.
Calculated amount of nitrogen for one-half that of uramino-acid .....	1.3 "

*Injection of  $\alpha$ -Ureidoisobutylacetic Acid.*

A cat weighing 2.4 kilos received 5 gm. of urethane by stomach tube and 5 gm. of  $\alpha$ -ureidoisobutylacetic acid<sup>2</sup> intravenously during the course of  $1\frac{1}{2}$  hours. The urine was collected as described in the experiment on phenylalanine injection. The collections were made over a 10 hour period and the material was examined for uramino-acid as in the previous experiment. A total of 350 cc. of material was obtained and extracted in portions. A considerable quantity of white crystalline substance separated during extraction and crystallized from the aqueous residues before concentration.

A yield of 1.87 gm. of substance was obtained on concentration of the combined aqueous residues.

The material melted at 208° (uncorrected) and gave off amino nitrogen before treating with hydrochloric acid. Isobutylhydantoin made from the uramino-acid melted at 212° (uncorrected) and gave off no amino nitrogen.

		NH <sub>2</sub> -N		
		Reaction time.	Found.	Theory for 50 per cent N.
mg.		hr.	mg.	mg.
19.0	Uramino-acid.	$\frac{1}{2}$	1.57	1.53
4.6	Hydantoin.	$\frac{1}{2}$	0	0

The mother liquor from the crystallization of uramino-acid was made alkaline with normal sodium hydroxide boiled to drive off ammonia and made up to 100 cc. Amino nitrogen determinations indicated the presence of an additional amount of uramino-acid of approximately 0.1 gm.

<sup>2</sup> Made from active leucine.

## CONCLUSION.

1. A method has been devised by which one of the disturbing factors in the study of uramino-acids in relation to their biological significance has been minimized, that is the interaction of amino-acid and urea, and Marshall's urease method for the destruction of urea by urease has been applied to a procedure where speed of decomposition with minimum analytical procedure was of especial importance.

2. The Van Slyke method for determining amino nitrogen has been applied to a new series of substances as a means for their identification and study. When associated with the fact that the anhydrides formed by the action of hydrochloric acid on these substances are not decomposed by nitrous acid to give amino nitrogen, the amino nitrogen of uramino-acids may be differentiated from that of amino-acids.

3. Small amounts of several uramino-acids when added to urine have been recovered.

4. The presence of preformed uramino-acid has not as yet been demonstrated in biological fluids following the injection of amino-acids.

5. Uramino-acids have been administered intravenously and recovered from the urine of the cat.

The writer wishes to thank Miss Marion Sweeney for her assistance in the experimental work.

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# ON A SOURCE OF ERROR IN THE USE OF PICRIC ACID IN COLORIMETRIC ESTIMATIONS IN BIOLOGICAL FLUIDS.

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(Received for publication, September 25, 1918.)

Critical studies of picric acid as used for the quantitative colorimetric determination of creatine and creatinine in blood (1) have been made by McCrudden (2), Hunter and Campbell (3), Folin and Doisy (4), Wilson and Plass (5), Greenwald and McGuire (6), and Denis (7) and suggestions made for modifying the procedure.

In carrying out blood sugar determinations by the Benedict modifications (8) of the Benedict-Lewis method for blood sugar determinations, the following observations were made upon picric acid.

A sample of Baker's chemically pure picric acid was purified by the method suggested by Folin (4). The material was divided into two portions, one put away in a dark closet in a brown bottle, dry (Picric Acid I), the other in the same closet in a colorless bottle with a little moisture (Picric Acid II). When these picric acids were next examined some 10 to 12 months later, the sample contained in the dark bottle was used for sugar determinations. A picric-picric acid solution was made up from the second portion in the colorless glass bottle. This solution failed to precipitate protein in the blood samples in the usual way. Apparently less coagulum was formed and the filtrate was more highly colored. A new sample of Baker's chemically pure moist picric acid, Picric Acid III, was made into a picric-picric acid solution as before and compared with the two former solutions in blood sugar determinations. Each picric-picric acid solution was used also to prepare a glucose standard and compared with a permanent standard of picramic acid.



TABLE I.  
*Colorimetric Readings on Dog's Blood.*

	Blood specimen.	Glucose solution.	Permanent standard.
Picric Acid I { Same original } .....	10.5	15	15
" " II { sample. } .....	4.0	15	15
" " III.....	10.5	15	15

Picric Acids I and III melted at 122° and Picric Acid II at 120°.

TABLE II.  
*Folin's Color Test for the Purity of Picric Acid.*

	Readings after 15 min.		Readings after 24 hrs.	
	Without alkali.	With alkali.	Without alkali.	With alkali.
	mm.	mm.	mm.	mm.
Picric Acid II.....	10	8.0	10	8.0
" " III.....	10	9.0	10	9.0

The figures would indicate that Picric Acid II is slightly inferior to No. III, but neither acid is to be discarded from this test. For a pure picric acid Folin gives 20: 14.

*Experiment to Test the Acidity of Picric Acids II and III.*

Samples of both Picric Acids II and III were titrated with 0.1 N sodium hydroxide, using phenolphthalein as an indicator.

Saturated picric acid solution.	Gm. per 50 cc.	Cc. 0.1 N NaOH necessary to neutralize.
II	0.60	23.5
III	0.60	26.5

The protein of the blood sample was precipitated according to Wilson and Plass (5), and blood sugar determinations were run according to the method of Benedict (8) upon the protein-free neutral material.

*Colorimeter Readings.*

Picric-picrate solution.	Blood specimen.*	Permanent standard.
	<i>mm.</i>	<i>mm.</i>
I	14.5	15
II	14.9	15
III	14.4	15

\* 2 cc. of filtrate equivalent of 2 cc. of blood.

## CONCLUSION.

A chromogenic substance other than sugar is present in the blood which certain picric acid fails to precipitate. Solid picric acid after purification may undergo a change in its precipitating value for chromogenic substances in the blood. The formation of a deeper color on addition of alkali to picric acid solutions as tested by Folin does not account for the discrepancy of certain picric acids in blood sugar determinations. The precipitating value of picric acid must be determined before reliance may be placed upon color production in quantitative procedures for blood sugar.

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# A METHOD FOR THE ESTIMATION OF POTASSIUM IN BLOOD.

By S. W. CLAUSEN.

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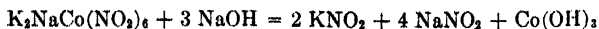
(Received for publication, September 9, 1918.)

This paper deals with an adaptation of the cobaltic nitrite method to the determination of potassium in small quantities of organic material.

The cobaltic nitrite method, as developed recently (1-5), includes three steps: (1) precipitation of the potassium as potassium sodium cobaltic nitrite,  $K_2NaCo(NO_2)_6 \cdot H_2O$ ; (2) oxidation of this precipitate in acid solution at boiling temperature with an excess of potassium permanganate; (3) titration of the excess of permanganate with oxalic acid.

In attempting to apply this method, it was at once evident that the small quantities of potassium involved (0.2 to 1 mg.) would necessitate the use of rather dilute  $KMnO_4$  solution. It was found that such dilute solutions, when boiled with acid, undergo appreciable decomposition. In order to eliminate this source of error, which is variable, use was made of an early and apparently abandoned step in the original method (6).

When the potassium sodium cobaltic nitrite is heated with dilute  $NaOH$ , all the nitrite groups are changed to sodium and potassium nitrite, and the cobalt is precipitated as insoluble  $Co(OH)_3$  which may be filtered off:



The nitrites may be estimated by titration with dilute potassium permanganate. The nitrite solution after cooling is acidified with  $H_2SO_4$  until about normal acidity is reached, and 0.02 N  $KMnO_4$  run in until a faint pink color persists for about  $\frac{1}{2}$  minute. The titration flask is then warmed on an electric

plate, and permanganate is slowly added until the color persists  $\frac{1}{2}$  minute at about  $70^{\circ}\text{C}$ . In a series of analyses of pure  $\text{NaNO}_2$ , in quantities varying from 0.5 to 5 mg. in 25 cc. of solution, the ratios of  $\text{KMnO}_4$  used to  $\text{NaNO}_2$  taken did not vary from each other more than 0.3 per cent. Furthermore,  $\text{NaNO}_2$  in such amounts as 0.5 to 5 mg. may be boiled with 1 per cent  $\text{NaOH}$  for 5 minutes without appreciable loss of reducing power for  $\text{KMnO}_4$ .

In the presence of normal acidity, at room temperature, the reducing power for  $\text{KMnO}_4$  remains apparently unchanged for 2 or 3 minutes, although the odor of the oxides of nitrogen is apparent. Of course at elevated temperature, the loss of reducing power is very rapid in acid solution.

These facts furnish the basis for the estimation of small quantities of potassium.

#### *Estimation of Potassium in Pure Solutions.*

The reagents are:

1. *Sodium Cobaltic Nitrite Reagent*.—This (7) is prepared from two stock solutions which keep well several months.

A. Cobalt nitrate.....	50 gm.
Glacial acetic acid.....	25 cc.
Distilled water to.....	100 "
B. Sodium nitrite.....	100 gm.
Distilled water to.....	200 cc.

6 parts A and 10 parts B are mixed. A rapid current of air is passed through the solution for several hours to remove the fumes of oxides of nitrogen. Then the dark brown fluid is kept on ice 2 days. Invariably some yellow precipitate forms, owing to traces of potassium and ammonium in the reagents. The filtered reagent keeps well for several weeks, if in a dark bottle in an ice box.

2. *Potassium Permanganate*.—This is prepared (8) by diluting 0.1 N  $\text{KMnO}_4$  to about 0.02 N and boiling under a funnel reflux condenser for 2 to 3 hours. After 1 to 2 days, the solution is decanted from the manganese oxides which have separated, and preserved in a cool dark place. Such solutions keep well several weeks.

### 3. *Standard Potassium Solutions.*

A. Standard, 1 cc. = 10 mg. potassium.

Pure KCl.....	9.546 gm.
HCl (concentrated).....	1 cc.
Water to.....	500 "

B.\* Standard, 2 cc. = 1 mg.

Standard A.....	25 cc.
HCl (concentrated).....	1 "
Water to.....	500 "

The potassium chloride is recrystallized several times, then fused. The hydrochloric acid is added to prevent growth of moulds.

The solution for analysis should contain 0.2 to 2.5 mg. of potassium. It is measured into a 50 cc. beaker and evaporated nearly but not quite to dryness, on a water bath. Two or three drops of glacial acetic acid are added, and 1 cc. of the sodium cobaltic nitrite reagent. Evaporation is continued until the mixture becomes syrupy. It is then cooled, and 4 to 5 cc. of water are added. One should not carry the evaporation too far, as insoluble brown compounds are formed, which are liable to be associated with too high results. The solution is cooled and 4 or 5 cc. of water are added.

The precipitate of potassium sodium cobaltic nitrite is filtered off on a Gooch crucible with a fairly thick asbestos mat. Filter paper must not be used, because during the subsequent boiling with 1 per cent NaOH, considerable reducing substance is formed from it. Just before the mat is sucked dry, a suspension of BaSO<sub>4</sub> is poured onto it. This not only fills up the larger pores, but serves as an excellent test of the mat. Before the water has all passed through the mat, the contents of the beaker containing the potassium sodium cobaltic nitrite are poured on. The beaker is rinsed with 4 to 5 cc. of cold water, and the rinsings are poured onto the nearly dry mat. Such washing, with 2 to 3 cc. of water, is repeated six times. The mat when almost dry is transferred to the beaker, and all adhering particles are washed from the crucible to the beaker with 9 to 10 cc. of water. 1 cc. of 10 per cent NaOH is added, and the contents of the beaker are heated to boiling, then cooled. The dark brown mixture is made up to exactly 25 cc. and centrifuged. 20 cc. of the clear fluid are pipetted off into a 150 cc. Erlenmeyer flask, 5 cc. of

1:4  $\text{H}_2\text{SO}_4$  added, and titration with 0.02 N  $\text{KMnO}_4$  as described above is at once carried out. Blanks, run frequently, are usually small (0.05 to 0.10 cc.). The potassium value of the permanganate is determined by analysis of the standard solution B. This value should be determined frequently, but does not vary greatly from day to day. Calculations are based on the values thus obtained rather than on a theoretical factor.

As may be seen from Table I, the analysis of pure KCl solutions is satisfactory.

TABLE I.  
*Analysis of Pure KCl Solutions.*

Potassium taken, mg.....	0.25	0.50	0.75	1.00
0.02 N $\text{KMnO}_4$ required, cc.....	1.53	3.08	4.59	6.13
0.02 N $\text{KMnO}_4$ per mg. K, cc.....	6.12	6.15	6.13	6.13

*Estimation of Potassium in Blood.*

In applying this method to blood, it was thought that the salts present might interfere, although Green (9) had shown that this was not the case in applying the cobaltic nitrite method to urine. I have found that Ca, Mg, Fe, and phosphates, in quantities as great as would be met with in blood analysis, do not interfere with the accuracy of the determination when added to pure KCl solutions. Organic material is removed by a "wet" ashing process.

The blood is ashed in a  $200 \times 25$  mm. Pyrex glass tube with a mixture of nitric and sulfuric acids. For 2 cc. of plasma, or 1 cc. of blood, 5 cc. of a mixture of sulfuric acid, 1 part, nitric acid 20 parts, are sufficient. The tube arranged with a fume absorber as for a Folin micro-Kjeldahl determination is heated by a microburner, adjusted so that boiling is slow, for about  $\frac{1}{2}$  hour. Foaming and bumping can be largely prevented by the use of a short piece of platinum wire sealed through the bottom of the tube, serving as a heat-conductor and boiling focus. After  $\frac{1}{2}$  hour the excess of  $\text{HNO}_3$  is rapidly evaporated. The remaining drop of sulfuric acid darkens considerably. At this stage, the flame is turned off, and the small amount of  $\text{HNO}_3$  condensed on the walls of the tube allowed to run back. If this is not sufficient to clear

up the  $\text{H}_2\text{SO}_4$ , two to three drops of  $\text{HNO}_3$  are added and boiling is resumed until the acid is colorless, when the tube is cooled.

The oxidation mixture is washed into a 50 cc. beaker, made alkaline to phenolsulfonephthalein with 10 per cent  $\text{NaOH}$ , and evaporated to dryness on the water bath. A few drops of glacial acetic acid are added, until the mixture is acid; then 1 cc. cobaltic nitrite reagent is added, and evaporation continued until crystals

TABLE II.  
*Recovery of Potassium Added to Serum.*

	mg.
1. Serum alone.....	0.61
Added K(as KCl).....	1.00
Total.....	1.61
Found.....	1.62
2. Serum alone.....	0.70
Added K(as KCl).....	1.00
Total.....	1.70
Found.....	1.70

TABLE III.  
*Potassium Determination in Blood, Expressed as Mg. per 100 Cr.*

Diagnosis.	Plasma.	Whole blood.
Circumcision.....	52.8	200
Nephritis (?).....	67	222
Diphtheria.....	65	
Pott's Disease.....		178
Nephritis.....		165
Cystitis; bladder stone.....	117*	234
Intestinal obstruction.....	89.5	265
Pyelitis.....	70.5	290
Alimentary intoxication.....	71.8	143

\* Slight hemolysis had occurred.

of  $\text{Na}_2\text{SO}_4$  appear. The mixture is then cooled. Dehydration by means of the  $\text{Na}_2\text{SO}_4$  results in very complete precipitation of the  $\text{KNa}_2\text{Co}(\text{NO}_2)_6$ . From this point the determination is carried out as for pure solutions already described.

That potassium added to blood may be recovered is evident from Table II. A few results obtained with plasma and whole blood are given in Table III.



In conclusion, it is to be emphasized that the reagents must be tested for the presence of potassium. One sample of c. p. NaOH purified by alcohol contained nearly 0.05 per cent potassium. The sodium citrate used as an anticoagulant must also be tested for the presence of potassium.

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## CONDUCTIVITY AS A MEASURE OF PERMEABILITY.

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(Received for publication, October 17, 1918.)

The method of electrical conductivity has been used by the writer to investigate the permeability of protoplasm. In this work it has been assumed that we can measure the permeability of protoplasm by determining the electrical conductivity of the tissues. But it may be objected that what we investigate by this method is not the protoplasm but only the non-living intercellular material by which the masses of protoplasm (cells) are separated in the tissue.

It is obvious that when a current passes through the tissue a considerable part of it must flow in the intercellular substance. Since this has a much lower resistance than the living protoplasm, the question arises whether any of the current passes through the protoplasm.

It might appear that this question could be settled by killing the protoplasm with agents which do not affect the intercellular substance and observing whether the conductivity continued to show the marked changes which it undergoes in the living tissue under the influence of certain reagents such as NaCl and  $\text{CaCl}_2$ . The results of such experiments show that after killing the cells the conductivity no longer changes when NaCl and  $\text{CaCl}_2$  are applied, but the question still remains whether the killing agent has not in some way altered the intercellular substance, so that its conductivity no longer changes as before.

In the case of *Laminaria* (which furnished the material for the experiments which have hitherto been described) the question could not be settled as completely as was desirable because the chemical behavior of the intercellular substance was not sufficiently known. Therefore it seemed desirable to repeat the experiments

with plants whose intercellular substance consists of cellulose and to kill the tissues by means which are known to produce no irreversible changes in the properties of cellulose. For this purpose experiments were made with a green marine alga (*Ulva*) and with a marine flowering plant (*Zostera*) both of which have cellulose walls.

The means employed for killing were various, including partial drying, treatment for a few hours with 25 per cent alcohol, exposure for a few minutes to sea water at 40°C., as well as placing for several hours in a solution of NaCl of the same conductivity as the sea water.

All these methods of killing produced similar results in that the conductivity rose to a constant value (indicating death) and was not thereafter affected by exposure to reagents which produce great alterations in the conductivity of living tissue.

We must therefore conclude that the alterations of conductivity observed in living tissue are due to changes in the protoplasm and not to changes in the non-living intercellular substance.

A very striking proof of this consists in adding to the sea water solid  $\text{CaCl}_2$  (or a strong solution of  $\text{CaCl}_2$ ). This greatly increases the conductivity of the solution and consequently of the intercellular substance which is freely imbibed with the solution. It might therefore be expected that the conductivity of the tissue would be increased. Just the contrary is the case. Although the conductivity of the intercellular substances increases, this is more than offset by the decrease of conductivity of the protoplasm due to the direct action of the calcium on the living protoplasm. The result is a decrease of the conductivity of the tissue as a whole. This decrease does not occur with tissue killed by the agents described above.

Another striking piece of evidence is found in the fact that the temperature coefficient of the electrical conductivity of living tissue differs from that of dead tissue. This could not be the case if the living protoplasm did not conduct a part of the current.

We must therefore conclude that while a part of the current flows in the intercellular substance another part flows through the protoplasm itself and that the variations in conductivity are due to changes in the living protoplasm and not to changes in the intercellular substance (unless cases should be found where ma-

terial killed as described above shows variations in conductivity similar to those found in living tissue).<sup>1</sup>

If, in spite of this demonstration, the question be raised whether determinations of conductivity really measure the permeability of the protoplasm, it may be answered that this method gives results of the same character as the method of plasmolysis, which has been generally relied upon for trustworthy information regarding permeability. In addition, the results obtained by the electrical method are in complete agreement with those obtained by a variety of other methods of measuring permeability, such as that of tissue tension, exosmosis, and diffusion through membranes of living tissue.<sup>2</sup>

This general agreement must mean that the electrical method measures permeability just as the other methods do.<sup>3</sup> It is, however, more accurate as well as more convenient. It enables us to make determinations at much shorter intervals than other methods. This not only permits the detection of rapid changes in permeability which would otherwise escape observation, but it enables us to determine with precision the time curve, which is indispensable in the study of dynamics.

<sup>1</sup> It is necessary to make sure that during the experiment the intercellular spaces should not change in size (as by the contraction of the protoplasm) or in content (as by the displacement of gas by liquid). This source of error was carefully guarded against in the experiments here described.

<sup>2</sup> Cf. Brooks, S. C., *Proc. Nat. Acad. Sc.*, 1916, ii, 569.

<sup>3</sup> The method may be applied to intact organisms as well as to pieces of tissue. Experiments with intact fronds of *Laminaria* give the same results as those made with pieces cut from the fronds.



## NOTE ON THE EFFECT OF DIFFUSION UPON THE CONDUCTIVITY OF LIVING TISSUE.

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(Received for publication, October 17, 1918.)

The writer has shown that electrolytes may be divided into two classes with respect to their effect on the electrical conductivity of living tissues: (1) those which produce a rise in resistance followed by a fall, and (2) those which produce only a decrease in resistance. Salts with bivalent and trivalent cations belong in the first class while those with monovalent cations belong in the second.

Recently some apparent exceptions to this rule have been met with. The behavior of certain substances is anomalous in three respects: (1) Although they are monovalent salts they produce a rise in resistance, (2) the rise is unusually brief in duration, (3) it occurs in dead as well as in living tissue.

As an example we may take an experiment with artificial sea water. This was made by mixing salts in the following molecular proportions: 1,000 NaCl, 78 MgCl<sub>2</sub>, 38 MgSO<sub>4</sub>, 22 KCl, 20 CaCl<sub>2</sub>, and adding sufficient water to make the electrical conductivity of the solution the same as that of sea water. When tissue of *Laminaria* was transferred from this to an artificial sea water (of the same conductivity as sea water) in which RbCl was substituted for NaCl, there was a sharp rise in resistance which disappeared in a few minutes. On transferring to artificial sea water with NaCl in place of RbCl, there was a drop in resistance.

When the tissue was transferred from artificial sea water made with NaCl to one (of the same conductivity as sea water) in which LiCl was substituted for NaCl, there was a drop in resistance; on replacing in artificial sea water made with NaCl there was a rise.

Similar effects were observed on dead tissue, but here, as was to be expected, the changes were not so great.

It is evident that these effects are due to diffusion. On transferring from artificial sea water made with NaCl to one in which RbCl takes the place of NaCl, the molecules of NaCl diffuse out of the tissue more rapidly than the larger molecules of RbCl can diffuse inward. Hence there is a temporary deficiency of salt in the tissue and the resistance accordingly rises. On the other hand the smaller molecules of LiCl diffuse in faster than NaCl can diffuse out, causing a temporary excess of salt, which lowers the resistance.<sup>1</sup>

Any confusion which might be caused by disturbances of this sort may be easily avoided by control experiments made on dead tissue.

<sup>1</sup> If tissue is transferred from sea water to a solution of RbCl (of the same conductivity as sea water) no rise is observed, since the diffusion effect is more than counterbalanced by the increase in permeability caused by pure RbCl.

# THE PROTEINS OF THE PEANUT, *ARACHIS HYPOGÆA*.

## III. THE HYDROLYSIS OF ARACHIN.

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(Received for publication, October 17, 1918.)

The preparation of arachin,<sup>1</sup> the principal protein in the peanut, *Arachis hypogæa*, has been described in the first paper of this series. The procedure used in the hydrolysis of arachin and the isolation of the amino-acids is in general the same as that described in the hydrolysis of kafirin.<sup>2</sup>

It will be noted in the description of the experimental work that trouble was encountered in extracting the esters from the second esterification. This was finally overcome by removing the bases by means of phosphotungstic acid and esterifying again.

Foreman<sup>3</sup> has called attention to the fact that glutaminic acid readily forms pyrrolidone carboxylic acid in dilute acid or alkaline solutions. He has also shown that glutaminic acid can be formed from pyrrolidone carboxylic acid by hydrolyzing with hydrochloric acid. This information was used in recovering glutaminic acid from the distillation residue.

The percentage of the basic amino-acids was determined by Van Slyke's method as well as by the direct method of Kossel and Kutscher. The values obtained for arginine and histidine by both methods agreed closely. The Van Slyke method showed the presence of more lysine than we succeeded in isolating by the direct method. The results of the hydrolysis are given in Table I.

<sup>1</sup> Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916-17, xxviii, 77.

<sup>2</sup> Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1918, xxxvi, 323.

<sup>3</sup> Foreman, F. W., *Biochem. J.*, 1914-15, viii, 481.



TABLE I.  
*Hydrolysis of Arachin.*

	<i>per cent</i>
Glycine.....	0.00
Alanine.....	4.11
Valine.....	1.13
Leucine.....	3.88
Proline.....	1.37
Phenylalanine.....	2.60
Aspartic acid.....	5.25
Glutaminic acid.....	16.69
Serine.....	—
Oxyproline.....	—
Tyrosine.....	5.50*
Cystine.....	0.85†
Arginine.....	13.51†
Histidine.....	1.88†
Lysine.....	4.98†
Tryptophane.....	Present.
Ammonia.....	2.03
Total.....	63.78

\* By the colorimetric method of Folin and Denis.

† Basic amino-acids determined by Van Slyke's method.

#### EXPERIMENTAL.

500 gm. of arachin, equivalent to 466 gm. of the ash- and moisture-free protein, were hydrolyzed separately in two portions of 200 and 300 gm. each with a mixture of 600 cc. of concentrated hydrochloric acid and 600 cc. of water by boiling in an oil bath for 48 hours. The solution was diluted with water and filtered through norite. In this way the humin was removed and a solution obtained which was nearly colorless. After thoroughly washing the norite with hot water, the washings and the main filtrate were united, the resulting solution was concentrated, and the glutaminic acid separated in the usual way by saturating with hydrochloric acid gas. There were obtained from the two portions 29.00 and 45.16 gm. respectively of glutaminic acid hydrochloride. With the 22.88 gm. subsequently obtained from the esters the total glutaminic acid hydrochloride weighed 97.04 gm., equivalent to 77.76 gm. of glutaminic acid, or 16.69 per cent of the arachin.

The free acid obtained by decomposing the hydrochloride with an equivalent amount of normal potassium hydroxide was analyzed with the following results:

0.2593 gm. substance gave 0.3860 gm. carbon dioxide and 0.1440 gm. water.

	Calculated for $C_6H_9O_4N$ :	Found:
C.....	40.80	40.60
H.....	6.12	6.21

The filtrates from the glutaminic acid hydrochloride were united, concentrated to a thick sirup, and freed from water by evaporation with alcohol under diminished pressure. The residue of amino-acids was then esterified according to the method of Phelps and Phelps,<sup>4</sup> and the resulting esters were liberated from their hydrochlorides by dissolving in absolute alcohol and adding the calculated amount of sodium necessary to combine with the chlorine, as described in a previous publication.<sup>2</sup> The alcohol was removed from the esters by evaporation under diminished pressure and collected in a receiver containing hydrochloric acid so as to avoid any possible loss of esters by volatilization. The alcohol was saved for further examination. The sirupy residue of esters was then stirred with about a liter of ether, whereupon it soon changed to a stiff, plastic mass. The clear ether extract was removed by decantation. On stirring the semisolid residue with more ether, it gradually became friable and was reduced to a powder. This was extracted with fresh portions of ether several times. The ether extracts were dried over anhydrous sodium sulfate in the usual way. The powdery residue remaining after the extraction with ether was freed from zinc by means of hydrogen sulfide, and subjected to a second esterification. The esterified product on cooling formed a hard, wax-like substance which would not yield to the usual methods of handling the esters at this stage. As this condition of the residue was probably due to the relatively large amount of basic amino-acids present, the esters were hydrolyzed and the bases removed by precipitation with phosphotungstic acid. The solution thus freed from the bases was evaporated to a sirup, and the residual amino-acids were esterified in the usual way. The ether was removed from the esters by distillation at atmospheric pressure. 260 gm. of

<sup>4</sup> Phelps, I. K., and Phelps, M. A., *Am. J. Sc.*, 1907, xxiv, 194.

esters were obtained, 79 gm. of which were obtained from the second esterification.

*Alcohol Distilled from the Esters.*—The alcohol, which had been distilled from the esters after their liberation from their hydrochlorides with sodium ethylate and which had been acidified with hydrochloric acid, was evaporated to dryness and the residue boiled with water to hydrolyze the esters. After removing the chlorine with silver sulfate there were obtained 8.42 gm. of amino-acids which were added to a similar fraction obtained from the ether, which was distilled from the esters as described below.

*Ether Distilled from the Esters.*—The ether which was distilled from the esters was acidified with an absolute alcohol solution of hydrochloric acid and allowed to stand at 0° for about a week. There was no separation of glycine ester hydrochloride. The ether was distilled off and the residue boiled with water for 7 hours to hydrolyze the esters. After removing the chlorine with silver sulfate, the solution was evaporated to dryness. To this residue were added the amino-acids obtained from the alcohol distilled from the esters as described above. The united fractions of amino-acids were boiled with absolute alcohol to extract the proline. This alcoholic extract of proline was added to the proline solution obtained from the distilled esters. There were thus obtained about 17 gm. of amino-acids insoluble in alcohol. From this mixture were isolated by fractional crystallization 1.22 gm. of leucine, 9.90 gm. of alanine, and 5.88 gm. of a mixture containing alanine, valine, and leucine. This mixture was added to a similar one obtained from Fractions I and II of the distilled esters.

Analysis showed the leucine to have the following composition.

0.1634 gm. substance gave 0.3282 gm. carbon dioxide and 0.1451 gm. water.

	Calculated for $C_6H_{13}O_2N$ :	Found:
C.....	54.96	54.78
H.....	9.99	9.94

The alanine had the following composition.

0.2072 gm. substance gave 0.3090 gm. carbon dioxide and 0.1469 gm. water.

	Calculated for $C_3H_7O_2N$ :	Found:
C.....	40.41	40.67
H.....	7.92	7.93

The esters remaining after the removal of the ether were distilled in the usual way with the following result.

Fraction.	Temperature of the bath up to	Pressure.	Weight.
	°C.	mm.	gm.
I.....	100	10.0	17.5
II.....	108	0.8	36.5
III.....	135	1.0	33.5
Distillation residue.....			146.0

*Fraction I.*—This fraction was hydrolyzed with boiling water, the solution of amino-acids evaporated to dryness under reduced pressure, and the proline extracted with boiling absolute alcohol.

By systematic fractionation of the free amino-acids from water, and from water and alcohol, there were obtained from this fraction 0.71 gm. of leucine, 7.51 gm. of alanine, and 2.05 gm. of a mixture consisting chiefly of valine and leucine. This mixture was united with other similar mixtures obtained during the course of this analysis (see below). The presence of glycine could not be established. The leucine gave the following analysis:

0.1329 gm. substance gave 0.2680 gm. carbon dioxide and 0.1198 gm. water.

	Calculated for $C_6H_{13}O_2N$ :	Found:
C.....	54.96	55.00
H.....	9.99	10.09

Analysis showed the alanine to have the following composition.

0.1445 gm. substance gave 0.2130 gm. carbon dioxide and 0.1050 gm. water.

	Calculated for $C_3H_7O_2N$ :	Found:
C.....	40.41	40.20
H.....	7.92	8.13

*Fraction II.*—The esters of this fraction were hydrolyzed by boiling with water until the alkaline reaction to litmus had disappeared. After removing the proline by extraction with absolute alcohol, a residue of amino-acids insoluble in absolute alcohol was obtained consisting of leucine and valine, from which by fractional crystallization 11.11 gm. of leucine were isolated. The

remainder formed an inseparable mixture of leucine and valine. To this mixture was added the fraction consisting of alanine, valine, and leucine obtained from the ether which was distilled from the esters, and also the valine-leucine mixture obtained from the distilled esters of Fraction I. These united portions were then subjected to the lead salt method of Van Slyke and Levene<sup>5</sup> for the separation of leucine and valine. There were thus isolated in the form of the lead salt 5.03 gm. of leucine. The lead leucine gave the following results on analysis.

0.2719 gm. substance gave 0.1767 gm. lead sulfate.

	Calculated for $\text{Pb}(\text{C}_6\text{H}_{11}\text{O}_2\text{N}_2)_2$	Found:
Pb.....	44.29	44.38

The filtrate from the lead leucine, after having been freed from lead with hydrogen sulfide, yielded by fractional crystallization 5.27 gm. of valine and 1.74 gm. of alanine. The valine crystallized in the characteristic lustrous plates, which by analysis were shown to have the following composition.

0.2678 gm. substance gave 0.5067 gm. carbon dioxide and 0.2282 gm. water.

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$	Found:
C.....	51.28	51.60
H.....	9.47	9.53

The alanine was analyzed with the following results.

0.1714 gm. substance gave 0.2547 gm. carbon dioxide and 0.1240 gm. water.

	Calculated for $\text{C}_3\text{H}_7\text{O}_2\text{N}$	Found:
C.....	40.41	40.53
H.....	7.92	8.08

All of the alcoholic extracts of proline were united. After filtering off a small amount of a substance which had separated on long standing, the solution was evaporated under reduced pressure to dryness. The residue was completely soluble in cold absolute alcohol. It was again taken down to dryness and the residue dissolved in water and made up to 500 cc. The total ni-

<sup>5</sup> Van Slyke, D. D., and Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1908-09, vi, 54.

trogen in this solution was 1.0687 gm. and the amino nitrogen 0.2885 gm. The difference, 0.7802 gm., is equivalent to 6.41 gm. of proline.

*Fraction III.*—The phenylalanine ester of this fraction was removed in the usual way by extraction with ether. It yielded 5.18 gm. of phenylalanine in the form of the hydrochloride. The free phenylalanine gave the following analysis.

0.2032 gm. substance gave 0.4862 gm. carbon dioxide and 0.1219 gm. water.

	Calculated for $C_9H_{11}O_2N$ :	Found:
C.....	65.45	65.26
H.....	6.66	6.71

There were further obtained from this fraction 9.54 gm. of aspartic acid as the barium salt, and 2.47 gm. as the copper salt. The free aspartic acid, obtained by decomposing the barium salt with sulfuric acid, reddened but did not decompose at 300°C.

0.2065 gm. substance gave 0.2731 gm. carbon dioxide and 0.0978 gm. water.

	Calculated for $C_4H_7O_4N$ :	Found:
C.....	36.09	36.07
H.....	5.26	5.30

The copper aspartate when recrystallized from water separated in the characteristic sheaves of needles.

0.1729 gm. air-dried substance gave 0.0501 gm. copper oxide.

	Calculated for $C_4H_5O_4N$ Cu. $4\frac{1}{2}H_2O$ :	Found:
Cu.....	23.07	23.15

*Distillation Residue.*—The undistilled portion of the esters was shaken with a mixture of ether and water to remove the phenylalanine. On standing there separated from the ether solution about 3.5 gm. of diketopiperazines which were filtered off, the ether being allowed to evaporate spontaneously. The residual esters were hydrolyzed by heating on a steam bath with concentrated hydrochloric acid. About 2 gm. of a black tarry substance separated which was removed by extraction with ether. The acid solution, after having been decolorized with norite, was concen-

trated and yielded 5.40 gm. of phenylalanine hydrochloride. The free phenylalanine, obtained by decomposing the hydrochloride with ammonia, gave the following results when analyzed.

0.1576 gm. substance gave 0.3766 gm. carbon dioxide and 0.0957 gm. water.

	Calculated for $C_9H_{11}O_2N$ :	Found:
C.....	65.45	65.17
H.....	6.66	6.79

The filtrate from the phenylalanine hydrochloride after having been freed from chlorine and the solution boiled with copper carbonate, yielded 0.66 gm. of copper aspartate which crystallized from water in the characteristic sheaf-like crystals.

There were further obtained from the filtrate from the copper aspartate, after having removed the copper with hydrogen sulfide, 0.3 gm. of tyrosine and 2.5 gm. of phenylalanine.

The aqueous solution remaining after shaking the distillation residue with ether and water was boiled with barium hydroxide to hydrolyze the esters. The barium was removed quantitatively with sulfuric acid and the solution concentrated. After filtering off 3.98 gm. of tyrosine which had separated, the solution was saturated with hydrochloric acid gas, but on standing for several days at 0° no glutaminic acid hydrochloride separated. The hydrochloric acid was removed by evaporation under reduced pressure and finally with silver sulfate, and the solution boiled with copper carbonate. On standing there crystallized 8.18 gm. of copper aspartate.

0.2084 gm. substance gave 0.0609 gm. copper oxide.

	Calculated for $C_4H_5O_4N$ Cu. $4\frac{1}{2}$ H <sub>2</sub> O:	Found:
Cu.....	23.07	23.35

The filtrate from the copper aspartate, after removing the copper with hydrogen sulfide, gave a large precipitate on addition of an excess of phosphotungstic acid. After the solution was freed from the basic amino-acids in this way, it yielded 2.0 gm. of tyrosine, 3.35 gm. of glutaminic acid hydrochloride, 3.36 gm. of copper aspartate, and a considerable amount of a clear, yellow sirup from which on long standing nothing separated. It seemed very probable that considerable of the glutaminic acid originally

present in the distillation residue might have been converted into pyrrolidone carboxylic acid, by having been subjected to the various steps in the foregoing analysis. Foreman<sup>8</sup> has shown that the glutaminic acid can be almost completely recovered by hydrolyzing the pyrrolidone carboxylic acid by boiling with 20 to 25 per cent hydrochloric acid. The above mentioned sirup was accordingly boiled with 25 per cent hydrochloric acid for about 19 hours. The solution was then concentrated to about 75 cc., saturated with hydrochloric acid gas, and allowed to stand for a few days in a refrigerator. A large yield of crystals separated. There were thus obtained 19.53 gm. of pure glutaminic acid hydrochloride which melted with decomposition at 197°C. The free glutaminic acid, obtained by decomposing the hydrochloride with an equivalent amount of normal potassium hydroxide, gave the following results on analysis:

0.2758 gm. substance gave 0.4117 gm. carbon dioxide and 0.1556 gm. water.

	Calculated for $C_5H_9O_4N$	Found:
C.....	40.81	40.71
H.....	6.12	6.31

*Tyrosine*.—A quantity of arachin equivalent to 1 gm. of the ash- and moisture-free protein was hydrolyzed by boiling with 25 cc. of 20 per cent hydrochloric acid for 11½ hours. The solution was decolorized with norite, made up to 100 cc., and the tyrosine determined colorimetrically according to the method of Folin and Denis.<sup>6,7</sup> The tyrosine found was equivalent to 5.50 per cent of the arachin.

*Aspartic Acid*.—50 gm. of arachin, equivalent to 46.62 gm. of ash- and moisture-free protein were hydrolyzed by boiling with 200 cc. of 20 per cent hydrochloric acid for 40 hours. The aspartic acid, together with some glutaminic acid, was separated from the other amino-acids by precipitation of their calcium salts according to the method of Foreman.<sup>8</sup> There were finally isolated in pure condition 5.42 gm. of copper aspartate. This is equivalent to 2.61 gm. of aspartic acid or 5.61 per cent of the arachin. The

<sup>6</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 245.

<sup>7</sup> Johns and Jones, *J. Biol. Chem.*, 1918, xxxvi, 319.

<sup>8</sup> Foreman, *Biochem. J.*, 1914-15, viii, 463.



copper aspartate crystallized from water in the characteristic sheaves, and gave the following results on analysis:

0.2061 gm. substance gave 0.0594 gm. copper oxide.

	Calculated for $C_4H_6O_4N\ Cu. \frac{4}{3} H_2O$	Found:
Cu.....	23.07	23.03

The total aspartic acid isolated from the distilled esters was 3.84 per cent, which is 1.77 per cent less than that obtained by the above method.

*Basic Amino-Acid.*—The bases had been previously determined by Van Slyke's<sup>9</sup> method. The bases were also determined by the direct method of Kossel and Kutscher. This determination gave 12.54 per cent of arginine, 2.13 per cent of histidine, and 1.72 per cent of lysine.

The values obtained for arginine and histidine agree well with those found by Van Slyke's method, while the value found for lysine is much lower.

The lysine was determined by weighing pure lysine picrate. A sirupy residue was left from which more lysine picrate could not be crystallized. This accounts in part for the discrepancy between the results obtained by the different methods.

<sup>9</sup> Johns and Jones, *J. Biol. Chem.*, 1917, xxx, 33.

# THE INFLUENCE OF PROTEIN FEEDING ON THE CONCENTRATION OF AMINO-ACIDS AND THEIR NITROGENOUS METABOLITES IN THE TISSUES.

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(Received for publication, October 8, 1918.)

In the last few years much information has been secured in regard to the metabolism of the proteins, due largely to the elaboration of accurate methods for the determination of amino-acids and their nitrogenous metabolites. As the result of many excellent and highly significant experimental researches, at least the general features of the method by which the mammalian organism utilizes the energy content of protein material have been revealed. The true significance of the amino-acids in relation to protein utilization and protein requirements is now known, and consequently the purpose of the extensive hydrolytic cleavage of proteins in the gastrointestinal tract is no longer ambiguous. There is now no reason to believe that the amino-acids undergo any appreciable change during their passage through the absorptive membranes of the intestines into the blood and lymph, and their ready access in greater or less amounts to all the tissues of the body cannot be doubted. Their subsequent catabolism is known to yield, on the one hand, a series of nitrogenous substances, whose identities and transformations have been determined experimentally with a large measure of success. On the other hand, the residual non-nitrogenous substances simultaneously produced have in the main eluded direct experimental research *in vivo*, a circumstance the more unfortunate, since they contain practically all of the physiologically available energy of the original amino-acids. The identity and the possible transformations to which these latter substances are subjected have of necessity been investigated by indirect methods. Needless to say, the interpreta-

tion of results secured by such methods is not simple. However, from the sum total of experimental data thus obtained certain inferences may be drawn concerning the general courses of breakdown of the non-nitrogenous residues of the various amino-acids, inferences which may reasonably be assumed to possess a high degree of probability.

There are, naturally, some phases of amino-acid catabolism concerning which the experimental evidence obtained is insufficient or even conflicting. For example, the question of the effect of protein feeding on the concentration of amino-acids in the tissues, and of certain of their nitrogenous metabolites, seems still unsettled. A somewhat detailed and critical review of the experimental evidence on this point is in order before considering the additional data to be reported in this paper.

It has been shown repeatedly by many experimental investigations that the amino nitrogen content of the blood may be doubled or even tripled during the digestion of a heavy protein meal, so that as regards the blood there can be no question that protein feeding increases its amino-acid concentration. As regards the muscle tissue, the evidence is not so clear.

Folin and Denis, in their pioneer investigations on protein metabolism, always obtained an increase in the non-protein nitrogen of muscle following the injection of amino-acids, amino-acid digestion mixtures, or Witte's peptone into a ligated loop of intestine, provided that such materials were readily absorbed. Furthermore, the increases thus obtained could not be accounted for by increases in urea or ammonia, and presumably, therefore, were due to the absorption of amino-acids by the muscle tissue from the blood. However, it cannot, of course, be concluded with certainty from these experiments that during the relatively slow liberation of amino-acids in normal protein digestion the amino-acid content of the muscles would increase, though certainly this evidence establishes a degree of probability to that effect.

The experiments of Van Slyke and Meyer along the same general lines are capable of yielding more definite information, since they determined directly the amino-acid nitrogen of their samples by the now standard nitrous acid method. However, most of their analyses were made on the tissues of dogs after the injection

into the venous blood of amino-acids and protein digestion mixtures. Only a relatively few experiments were performed on dogs during normal protein digestion. The experiments on the injection of amino-acids clearly demonstrated the following:

1. The tissues rapidly absorb amino-acids from the blood when their concentration in that fluid is increased. An equilibrium is reached when the tissues contain roughly about ten times the percentage of amino-acid present in the blood. This process is not a mere diffusion, though the tissues do not appear to be in chemical union with the absorbed amino-acids.

2. Muscle and kidney tissue retain all of the amino-acids thus absorbed for 3 or even 4 hours. Apparently there is a latent period of several hours duration between the taking up of amino-acids by the muscles from the blood and the catabolism of the excess amino-acids. The liver, on the other hand, although capable of absorbing more amino-acids per unit of weight than any other organ or tissue examined, does not retain them for any length of time: in 3 or 4 hours the excess amino-acids had disappeared. Furthermore, there seemed to be no reason for assuming that they had been transferred to any other organ or tissue, and since, simultaneously with their disappearance from the liver, there was a rise in the urea content of the blood, the conclusion was drawn that the liver catabolized amino-acids at a very much greater rate than the muscles, and that at least a part of the nitrogen thus formed is converted into urea.

These are the most important facts and conclusions resulting from the experiments on the intravenous injection of amino-acids into dogs. From experiments on fasting dogs and on dogs digesting a heavy protein meal, two results of outstanding importance were obtained: (1) In dogs which, after 48 hours fasting, were given heavy meals of meat, in all cases the urea content of the blood rose almost immediately after the meat was consumed. Roentgenograms showed, in fact, that the rise in urea was simultaneous with the passage of the first particle of chyme from the stomach to the duodenum. (2) A comparison of the tissues of fasting dogs with those of dogs killed a few hours after consuming fresh meat showed that neither the livers nor other tissues of the fed animals contained a definitely greater store of amino-acids than did the tissues of the fasting animals.

From these somewhat remarkable results it was concluded that, unreasonable as it may appear, the organism does not wait until it has absorbed sufficient protein digestion products to meet its immediate requirements and thereafter begin to turn the surplus into urea. The very beginning of absorption stimulates the urea-forming function into activity, this initial urea formation being attributed to the liver. It was also concluded that the concentration of amino-acids in the body is so well regulated, presumably by the liver, that no great fluctuations ordinarily occur, even after heavy consumption of protein. "The digesting dogs must have either destroyed or condensed into protein practically all the amino-acids which they absorbed, and have done so at a rate which was nearly parallel with that of absorption."<sup>1</sup>

The above interpretation of the two experimental results just stated seems open to question. In the first place the formation of urea by the liver from ammonium salts arising from the hydrolysis of protein in the stomach and intestine is not considered. The increase observed in the concentration of urea in the blood cannot with certainty, therefore, be ascribed to the deamination of amino-acids, and such an interpretation may even be considered improbable in view of the close coincidence observed between the appearance of surplus urea in the blood and the first passage of chyme from the stomach to the duodenum. In all probability no amino-acids are liberated during the gastric digestion of proteins, at least in any but negligible quantities, while ammonia is undoubtedly one of the products of peptic digestion and is produced in considerable quantities from most proteins. Hence any urea formed by the liver immediately after the entrance of chyme into the duodenum more probably originated from ammonium salts than from amino-acids. The ability of the liver to convert rapidly ammonium salts of organic acids into urea is well recognized, while its ability rapidly to deaminize amino-acids and convert the resulting ammonia to urea is not so well established, and indeed has been denied by some investigators.

Hence the rate of increase of urea in the blood during protein digestion can give no sure clew to the rate of amino-acid catabo-

<sup>1</sup> For a very complete discussion of Van Slyke's experimental data and the conclusions deduced therefrom, see Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

lism, nor to the time at which this catabolism is initiated. The liver is probably competent to keep the concentration of ammonia in the systemic blood and in the other tissues within very narrow limits under normal conditions of metabolism, by conversion of ammonium salts into urea; that it is competent to do likewise with the concentration of amino-acids is less certain. In fact, if the liver possessed such a capacity, many phases of protein utilization, especially in the growing animal, would be difficult to understand. It may be mentioned, also, that there exists an unexplained contradiction between the results of Van Slyke, which indicate the formation of urea almost immediately after the ingestion of protein, and those of Folin and Denis, which indicate a lag of  $\frac{1}{2}$  to 1 hour between the time of injection of amino-acid mixtures into a ligated loop of intestine and an increase in the concentration of urea in the blood.

As regards the second result of the experiments on fed and fasting dogs, which indicated no essential differences in the concentration of amino-acids in the tissues of fasting dogs and of dogs killed at the height of protein digestion, the published data on this point are too few to be particularly convincing.<sup>2</sup> Analyses have been reported by Van Slyke and Meyer of the amino-acid content of the tissues of seven dogs, one killed 5 hours after a meal of 1 pound of fresh meat, and the others killed after fasting 18 hours, 20 hours, and 2, 4, 6, and 12 days. It will be noticed that the comparison must be made between one fed dog, and six dogs fasted for different intervals, all of which, however, were killed in the "postabsorptive period." The data for the fasted dogs are extremely variable among themselves and for no single tissue is there a consistent progressive increase or decrease in amino-acid content with the length of the fasting period. It is true that the data for the single fed dog are not to be distinguished from those for the other animals, but it seems obvious that the data are statistically insufficient to justify drawing positive conclusions. If animals killed 5 hours after a meat meal exhibit as great a variability in the amino-acid content of their tissues as the six fasted dogs, it is quite possible that the one fed dog actually analyzed may have been exceptional in this respect, a surmise some-

<sup>2</sup> Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913-14, xvi, 231.

what fortified by the fact that the amino-acid content of the blood of the fed dog was no higher than that of the dog fasted for 18 hours and only slightly higher than that of the dog fasted for 4 days. This relation is quite different from that reported in an earlier paper by the same investigators,<sup>3</sup> in which it is shown that the amino-acid content of the blood of two dogs killed 5 hours after a meal of 1 kilo of fresh beef is about double that of the same dogs before the meal and following a 24 hour fast.

Passing from the data to the interpretation, *i.e.* that digesting dogs must destroy or condense into protein all amino-acids, which they absorb, at a rate parallel to that of absorption, the objection may be raised that such an interpretation is not consistent with the numerous results obtained on dogs following the injection of amino-acids intravenously. These experiments, as mentioned above, showed clearly that the muscles and kidneys retain in undiminished amount the excess amino-acids absorbed from the blood, for as long as 4 hours. No reason is apparent why, in the one case, amino-acid catabolism should be so rapid in the muscles, while, in the other case, it is so long delayed. On the basis of the results obtained in the injection experiments, and of the demonstrated fact that during protein digestion the amino-acid content of the systemic blood is notably increased, one should expect that the amino-acid concentration in the muscles would increase also to an extent corresponding to the amino-acidemia and its duration, and that this increased concentration would persist for several hours. As a matter of fact, Folin and Denis obtained results that may reasonably be interpreted as indicating an increased amino-acid concentration in the muscles following the injection of amino-acid mixtures into the intestine.

In order to understand the extent to which protein nutriment is accessible to the tissues of the body, and the function of the liver in regulating the amino-acid concentration of the tissues, additional information is needed. If the deaminizing function of the liver is as vigorous as Van Slyke assumes, no appreciable change in the amino-acid concentration of the tissues would occur during protein digestion, and as a consequence only a negligible portion

<sup>3</sup> Van Slyke and Meyer, *J. Biol. Chem.*, 1912, xii, 408.

of the amino-acids coming to the liver through the portal vein could be assumed to reach the other tissues of the body. Under these conditions, the growing animal, the animal recuperating from disease or inanition, and the pregnant or lactating female, would be placed under a great handicap in covering their protein requirement, unless a further assumption can be made that under these conditions the deaminizing function of the liver is in some way depressed to allow the tissues to obtain abundant protein nutriment. On the other hand, if the function of the liver is simply to prevent the amino-acid concentration of the blood and tissues from increasing to such an extent as to produce injurious results, that is, if the deaminizing function of the liver operated at full capacity only under the stimulus of an excessive amino-acidemia, then it would be expected that the tissues could at all times secure sufficient protein nutriment without being overloaded and injured, as could be demonstrated experimentally by the determination of moderate increases in the amino-acid concentration of the tissues during protein digestion.

The main purpose of the experiments to be reported in this paper was to determine whether or not an increase in the amino-acid concentration of the tissues actually occurred during protein digestion, and, second, to obtain information if possible, as to the rate of amino-acid catabolism, by investigating the changes in concentration in the tissues of the nitrogenous metabolites of the amino-acids, ammonia, and urea. The investigation was undertaken with the conviction that the accumulation of a considerable amount of data is requisite before satisfactory conclusions can be drawn; the problem is essentially a statistical one. As an additional illustration of the difficulties that may be encountered when the data are too few and too variable to warrant interpretation, reference may be made to an investigation by Miss Wishart, working in Lusk's laboratory, to determine the influence of meat ingestion on the amino-acid content of blood and muscle.<sup>4</sup> The muscle tissue of three fasting dogs was analyzed for total non-protein nitrogen and for urea. The results for non-protein nitrogen other than urea, presumably largely amino-acid nitrogen, were 113, 81, and 145 mg. per 100 gm. of tissue. For two dogs killed 3 hours after the ingestion of 1 kilo of meat, the correspond-

<sup>4</sup> Wishart, M. B., *J. Biol. Chem.*, 1915, xx, 535.



ing values were 115 and 132 mg., and for two dogs killed 5 hours after the meat meal, the values were 101 and 117 mg. While the conclusion drawn was that no positive increase in the amino-acid content of muscle occurs during protein digestion, the disparity existing among the figures for the fasting dogs undoubtedly introduces a considerable element of doubt as to the correctness of the conclusion. However, the work of Wishart, and of Van Slyke and Meyer has induced Lusk to change his theory as to the cause of the specific dynamic effect of the amino-acids. Convinced that no appreciable change in concentration of amino-acids in the tissues occurs during digestion, he now concludes that the amino-acids themselves cannot be the cause of the increased heat production, but that the first products of amino-acid breakdown, keto- or hydroxyl-fatty acids, are probably the cell stimulants.

#### *Methods.*

Albino rats were used in this investigation, since, with such small animals, the analysis of the entire carcass presents no particular difficulties. The plan of the experiment was to make analyses of animals killed after a fast of 24 to 48 hours, and, for comparison, of other animals killed at varying periods after the ingestion of a high protein meal. The ration fed to the latter animals consisted of casein (Eimer and Amend) 60 per cent, skim milk powder (Merrell-Soule) 10 per cent, and butter fat 30 per cent. On analysis the ration was found to contain 7.75 per cent of nitrogen. Considering the ingredients used, this nitrogen must have been practically all protein nitrogen.

The animals were all killed with ether, and opened immediately. The gastrointestinal tract was removed from cardia to rectum and thoroughly washed out with physiological salt solution. For most of the animals the liver, and occasionally the kidneys, was removed for separate analysis, together with a sample of muscle tissue weighing generally from 8 to 10 gm., taken from the hind legs which were practically stripped of muscle to provide the sample. For all rats analyzed, the bladder was empty of urine. The carcass and intestinal tract were analyzed together, after passage through a meat grinder. All samples were extracted with boiling ammonia-free water, acidified with acetic acid, according to the recommendation of Van Slyke.<sup>5</sup>

<sup>5</sup> Van Slyke, *J. Biol. Chem.*, 1913-14, xvi, 187.

Each sample was extracted eight times with approximately five times its weight of water. The carcass and intestines were analyzed for ammonia, amino nitrogen, and urea; the separate tissues, for ammonia and urea only. In the former case, ammonia was determined both by the distillation method of Van Slyke<sup>5</sup> and by simple aeration after the addition of potassium carbonate, simultaneously with the urea determination. For the liver, muscle, and kidney samples, only the distillation method was used, urea being determined in the extract freed of ammonia by that means. Amino nitrogen was determined by the nitrous acid method of Van Slyke,<sup>5</sup> always on ammonia-free material, and urea by the method of Van Slyke and Cullen,<sup>6</sup> using a vacuum pump and continuing the aeration for 1 hour. By repeated test, 1 hour was found to be more than sufficient to draw through the solutions 100 liters of air. For both the ammonia and urea determinations approximately 0.02 N sulfuric acid was used, back titrations being made with alkali of equal strength.

At first the amount of ration eaten by each of the fed rats previous to analysis was accurately weighed, but later no record was kept of this, since it was evident that the analytical results obtained bore no relation to the amount of food eaten, possibly because in all cases enough food was taken to keep the stomach well filled up to the time of killing. There seems to be no reason for believing that the amount of food taken into the stomach during a meal, bears any relation to the rate of passage of chyme into the duodenum, except perhaps in cases involving extreme gorging of the stomach.

All the above analyses were run on protein-free solutions. At first, the attempt was made to precipitate the non-coagulable proteins by *m*-phosphoric acid, but difficulties in filtration finally led to the use of five volumes of absolute alcohol for that purpose.<sup>5</sup> Tests on one rat extract indicated that the latter reagent gave not only a more easily filterable precipitate, but also a more complete precipitation. Thus, for one of the fasted rats, the nitrogen not precipitated by *m*-phosphoric acid amounted to 2.072 gm., while that not precipitated by alcohol was only 1.349 gm. At the same

<sup>5</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211; 1916, xxiv, 117.

time, the amino nitrogen in the two cases was practically identical; *i.e.*, 0.247 gm. from the *m*-phosphoric acid precipitation and 0.239 gm. from the alcohol precipitation. In another test on a fed rat, the total non-protein nitrogen by the *m*-phosphoric acid precipitation was 0.551 gm., and by the alcohol precipitation, 0.384 gm. The amino nitrogen in the former case was 102 mg., and in the latter case only 88 mg., an appreciable difference.

### *Results.*

The experimental data obtained on the main portions of the rat carcasses, in some cases exclusive of the special tissue samples, are given in Table I, expressed in mg. of nitrogen per 100 gm. of rat. The ammonia values thus expressed are fairly constant and indicate no clear distinction between fed and unfed animals. The urea values are more variable, and indicate clearly, as would be expected, that in the fed animals the concentration of urea in the tissues is distinctly greater than in the fasted animals. It is a fair conclusion, therefore, that an active amino-acid catabolism was occurring in those animals killed a few hours after feeding.

The amino nitrogen figures show a noticeable tendency to decrease from the earlier to the later experiments. This relation is especially well shown in the case of the fasting animals. For the ten animals killed in the postabsorptive period, there is a nearly continuous decrease from the first to the last. For the thirteen animals killed during active protein digestion, the decrease is marked from November to March, after which the values obtained are quite uniform. Another significant fact shown by Table I is that for rats killed in November there is a rather clear distinction between fed and unfed animals, the amino-acid concentration of the fed animals being uniformly higher than that for the unfed. For rats killed from March to June the difference is not so clear. While the fed rats still appear to have a somewhat higher amino nitrogen value, the average difference is within 10 mg. per 100 gm. of tissue. It may be noted here that the change from *m*-phosphoric acid to alcohol as a protein precipitant was made in January. Therefore, the high amino nitrogen values

obtained in November may have been due in part to this change in technique. It could not, however, account for the differences observed between the fed and fasted animals at that time.

TABLE I.

*Non-Protein Constituents of the Rat Carcasses. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.*

Date killed.	Fasted rats.					Fed rats.					
	No. of rat.	Body wt.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.	No. of rat.	Body wt. (unfed).	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.	Time since last meal.
1917		gm.	mg.	mg.	mg.		gm.	mg.	mg.	mg.	hrs.
Nov. 9	1	120.0	16	—	136	2	134.4	11	—	152	5
" 16	3	93.6	19	31	95	4	92.2	12	61	141	5
" 23	5	141.5	12	—	84	6	123.4	10	53	102	5
1918											
Jan. 15	7	239.6	12	21	102	—	—	—	—	—	—
Feb. 4*	8	211.5	9	18	46†	—	—	—	—	—	—
" 26	9	188.5	10†	28†	60†	—	—	—	—	—	—
Mar. 14	10	165.9	15	23	63†	11	168.2	14	30	60†	5
Apr. 11	—	—	—	—	—	12	154.3	12	35	54†	3
" 12	13	132.2	13	17	52†	—	—	—	—	—	—
" 16	—	—	—	—	—	14	171.5	10	21	53†	3
May 3	15	143.0	14	32	53†	16	135.7	12†	40†	63†	3
" 9	—	—	—	—	—	17	127.0	14†	25†	53†	1
" 10	18	172.0	12†	14†	46†	19	154.0	13†	24†	50†	7
June 10	20	138.0	—	9	44†	21	183.0	—	43	58†	3
" 11	—	—	—	—	—	22	190.5	—	35	56†	5
" 26	—	—	—	—	—	23	187.3	10	23	63†	3
" 26	—	—	—	—	—	24	142.0	12	33	59†	7

\* This rat was fed 5 hours before killing and ate the ration voraciously. Immediately after consuming an unusual quantity, however, it was evidently in great discomfort, experiencing difficulty in breathing. Upon opening the animal it was seen that the stomach was distended to capacity with food, and that the esophagus, as far up as the pharynx, was also distended with food. No evidence of the passage of food into the intestines was noted, so that for this reason as well as the low urea value subsequently determined, the animal should probably be considered as a fasting subject. Evidently the dyspnea due to the packed esophagus had inhibited all the digestive activities, muscular and chemical.

† These results were obtained by analysis of the carcass minus the liver, the muscle sample, generally 8 to 12 gm., and occasionally the kidneys.

The first possibility that presented itself as to the cause for the progressive changes observed in the amino nitrogen figures was that the age of the animal might be a determining factor in the amino-acid concentration of its tissues as well as the reaction to a protein meal. The rats used in the experiments reported above were obtained from a Massachusetts dealer and were a fairly uniform lot as regards size and apparently age also, being less than half grown when received in October. It seemed possible, therefore, that increasing maturity during the course of the investigation may have been the cause of the progressive change in the amino nitrogen data. To test this possibility, five young rats of the same litter, weighing from 50 to 60 gm., were killed and the entire carcasses analyzed as usual for ammonia, urea, and amino

TABLE II.

*Non-Protein Constituents of a Litter of Young Rats. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.*

Sex.	Weight, unfed.	Time since feeding.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.
	gm.	hrs.	mg.	mg.	mg.
Female.....	50.5	24+	16	22	80
Male.....	56.8	24+	15	24	80
Female.....	50.8	3	20	37	85
Male.....	60.5	3.5	21	41	102
Female.....	51.7	5.5	—	38	108

nitrogen. While the age of these animals had not been recorded, since at the time of their birth it was not the intention to use them in this work, they were a well nourished lot and probably of normal size for their age. The animals were killed on June 27, two of them after fasting for 24 hours, two of them from 3 to 3.5 hours after feeding the experimental ration, and one of them 5.5 hours after feeding. The results are given in Table II.

These figures reveal higher values in the young fasting animal for ammonia and amino nitrogen than in more mature fasting animals. In particular there is a distinct increase in all three forms of nitrogen for the fed as compared with the fasting animals. The value of 85 mg. of amino nitrogen for the rat killed 3 hours after feeding is possibly too low, since in the concentration

*in vacuo* of the solution for this determination, evaporation accidentally proceeded to dryness and the temperature of the bath rose to over 90°C. As Van Slyke<sup>5</sup> has shown, such an accident may lead to a loss of amino nitrogen. We may conclude therefore, that for these young rats the ingestion of a protein meal increases distinctly the concentration of amino-acids and of their nitrogenous metabolites in the tissues.

To confirm this point further, another litter of four rats, somewhat older than the preceding, was killed on July 18 and analyzed. Two of the rats had fasted 24 hours or more, while the other two had been given the experimental ration about 5 hours before. The results are shown in Table III.

TABLE III.

*Non-Protein Constituents of a Second Litter of Young Rats. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.*

Sex.	Weight, unfed.	Time since feeding.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.
	<i>gm.</i>	<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Male.....	95.0	24+	16	28	61
Female.....	89.0	24+	17	19	61
Male.....	76.2	5	29	27	70
" .....	73.5	5.5	---	40	79

These values reveal essentially the same conditions as those obtained with the other litter of young; while the amino nitrogen figures are lower than those given by the others, due probably to the more advanced development of these animals, there is still an appreciable increase in amino nitrogen due to feeding. The high urea value for the first rat is possibly correlated with the fact that the stomach still contained appreciable amounts of food residue, while the cecum was well filled. With all other fasted animals, the stomach was practically empty. The absence of a high amino nitrogen value also in this rat, may be due to the fact that the animal was apparently killed in the last stages of digestion, when the amino-acid catabolism in the muscles had been in active operation for several hours, had disposed of the excess of amino-acids accumulated in the first stages of digestion, and was deaminizing amino-acids at a rate equal to the absorption from the blood.

When the above analyses were completed, there was one more litter of young left for use in this experiment. It was, however, with some hesitation that these rats were taken, since they were very poorly nourished, had rough coats, and one was afflicted with badly infected eyes. They weighed from 17.7 to 24.2 gm., though they were considerably older than their weight would lead one to expect. In fact, at their age of 38 days these rats should have weighed from 50 to 55 gm. Their poor nutritive condition was due probably to two circumstances: first, the mother had been killed for experimental purposes before the young were properly weaned, and, second, the weather for some time had been extremely hot, and the temperature of the rat quarters had very often been over 102° F. However, with the idea of utilizing all available sources of information, the entire litter was killed for analysis on July 29. The entire litter had been fasted for 24 hours, at which time three were fed the experimental ration and subsequently killed at the end of 1.5, 3, and 3.5 hours. The

TABLE IV.

*Non-Protein Constituents of a Third Litter of Young Rats. Results Expressed in Mg. of Nitrogen per 100 Gm. of Rat.*

Weight, unfed.	Time since feeding.	Ammonia nitrogen.	Urea nitrogen.	Amino nitrogen.
gm.	hrs.	mg.	mg.	mg.
21.0	24+	13	26	71
18.8*	24+	20	67	91
23.8	1.5	16	28	68
24.2	3	14	22	51
17.7†	3.5	26	52	70

\* Dead an hour or more before extraction.

† Both eyes badly infected.

two remaining rats were killed last, without being fed; one was in an extremely weak condition, and the second died an hour or so before the extraction with boiling water could be started. The analytical results secured are given in Table IV.

These results are not at all in agreement with the others reported above, since no definite effect of the protein feeding is evident, even in the case of urea. The high urea and amino nitrogen values for the rat that had been dead for some time before analysis was started, indicate that postmortem changes had already set in. In accounting for the lack of agreement between these results and those secured on normal rats, it is possible that the poor nutritive condition of these animals had impaired their digestive and absorptive powers. Certainly their appetites were not normal, since they displayed no eagerness when food was offered to them and ate sparingly of it, quite contrary to our experience with the other rats. The data are presented simply to complete the record of all evidence collected on the points at issue.

The analytical results obtained on the livers, muscle tissue, and kidneys from the adult rats related only to ammonia and urea nitrogen. It was unfortunate that the amino nitrogen was not also determined, but with the methods used, the samples were insufficient for this purpose. The results are shown in Table V.

TABLE V.

*Non-Protein Constituents of Rat Tissues. Results Expressed in Mg. of Nitrogen per 100 Gm. of Tissue.*

No. of rat.	Digestive condition.	Weights of tissues.			Urea nitrogen per 100 gm. of			Ammonia nitrogen per 100 gm. of		
		Liver.	Muscle sample.	Kidneys.	Liver.	Muscle.	Kidney.	Liver.	Muscle.	Kidney.
		gm.	gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.
5	Fasting.	6.1	—	—	—	—	—	10	—	—
7	"	8.5	—	—	35	—	—	16	—	—
8	"	8.464	11.920	—	49	11	—	14	9	—
9	"	7.418	11.324	—	21	—	—	—	—	—
10	"	6.163	8.631	—	22	13	—	41	27	—
13	"	4.382	6.996	1.139	4	13	36	29	11	65
15	"	6.956	9.317	1.323	17	13	—	—	—	—
18	"	9.010	9.418	1.761	16	—	—	—	—	—
20	"	5.888	8.309	—	19	11	—	22	15	—
17	1 hr. after meal.	5.979	7.531	1.067	—	—	—	—	—	—
12	3 hrs. " "	6.232	7.735	1.338	22	25	44	33	22	80
14	3 " " "	8.420	10.014	1.501	22	16	116	16	16	132
16	3 " " "	7.723	7.747	1.356	—	—	—	—	—	—
21	3 " " "	7.337	20.794	—	25	18	—	21	9	—
23	3 " " "	8.587	10.545	—	29	23	—	22	16	—
6	5 " " "	5.4	—	—	—	—	—	11	—	—
11	5 " " "	7.361	10.410	—	34	24	—	23	25	—
22	5 " " "	7.493	11.688	—	26	20	—	16	17	—
19	7 " " "	5.612	8.911	1.268	—	—	—	—	—	—
24	7 " " "	6.207	8.597	—	21	18	—	25	19	—

The urea content of the liver is generally greater than that of muscle tissue, a fact confirming the general opinion that the liver is more active in urea formation than is muscle. The liver exceeds the muscles also in its concentration of ammonia, in most cases. Erratic figures for the kidney may be expected from the smallness of the sample and its variable content of urine.



## DISCUSSION.

The non-protein constituents of rat tissue are quite comparable in concentration with those of dog tissue, for example, a confirmation of the tacitly accepted opinion that mammalian metabolism is much the same, regardless of the particular species of animal under consideration. However, a fact discovered in this investigation and not noted in other work, so far as the author is aware, is that the ammonia and amino-acid concentration of young animals is greater than that of adult animals. Whether this relation represents simply a different anatomical make-up of the young animal, such as a greater proportion of viscera and a smaller proportion of muscle, than of the adult, or whether, in addition, it is concerned possibly in the more intense metabolism of the young, it is perhaps premature to say. The difference seems to be greater, however, than can be accounted for on anatomical grounds alone. Possibly the more rapid and general synthesis of protein in the tissues of the young animal may require at all times a greater concentration of amino-acids and ammonia upon which to draw, which may be assured by a greater avidity of the tissues for these substances and a less intense catabolism of them than in the adult.

Perhaps the most important and interesting result of the investigation is the evidence secured as to the effect of protein feeding on the amino-acid concentration of the tissues. Here again, the age of the animal seems to be a determining factor. In the vigorously growing animal, during protein digestion there is a distinct increment in the amino-acid content of the tissues, while as the rate of growth decreases this increment becomes smaller, until in the adult it is only of moderate or perhaps even insignificant dimensions. Apparently there is a relation between the amino-acid requirements of the tissues and their accessibility to the end-products of protein digestion. In explaining such a relation several possibilities suggest themselves: (1) The digestive and absorptive powers of the young organism are more vigorous than of the old, so that of a given protein ration, more is digested and absorbed in a given time, producing a higher amino-acidemia and, by this factor alone, a greater increase in the amino-acid concentration of the tissues. (2) The deaminizing function of the

tissues, especially of the liver, in the young animal is less intense than in the old, permitting a greater influx of amino-acids through the portal circulation and a more delayed catabolism in the tissues. (3) The tissues of the young animal possess a greater absorptive power for amino-acids than those of the adult, so that the equilibrium in amino-acid concentration between tissue and blood is considerably more than 10:1.

However the relation between amino-acid concentration of the tissues and the age of the animal may be explained, the fact remains that in the adult animal only an inconsiderable increase in amino-acid concentration during protein digestion has been demonstrated. It is quite possible that the large percentage of fat in the experimental ration impeded the emptying of the stomach to such an extent that the absorption of amino-acids was relatively slow, and that no considerable accumulation of amino-acids occurred in the blood, and consequently none in the tissues. However that may be, the results obtained confirm the viewpoint of Van Slyke for the grown animal. Whether the liver is the prime factor in the regulation of the amino-acid concentration of the tissues seems questionable, since amino-acids undoubtedly accumulate in the systemic blood during normal protein digestion, even when no considerable accumulation can be demonstrated in the tissues. The more plausible explanation seems to be that the tissues catabolize the amino-acids about as rapidly as they are absorbed from the blood.<sup>7</sup>

Some consideration of the significance of these facts on the problem of the cell stimulants concerned in the specific dynamic effects of amino-acids may not be out of place. The failure to demonstrate a considerable increase in the amino-acid concen-

<sup>7</sup> This interpretation, of course, seems inconsistent with the experimental results of Van Slyke and Meyer, indicating that when amino-acids are injected into the circulation, they accumulate to some extent in the muscles and are retained there for several hours unchanged. However, the constant high amino nitrogen figures observed in muscle for 3 or 4 hours after amino-acid injection may not indicate necessarily a total absence of catabolic activity. They might also be interpreted as meaning a slow catabolism in the muscles, compensated for by a replenishing of the store of amino-acids there from the liver and possibly other tissues. While the data obtained offer no particular support for such an interpretation, as Van Slyke and Meyer state, they are nevertheless consistent with it.

tration of the tissues of adult animals during protein digestion at a time when the specific dynamic effect of protein is being exerted does not necessarily rule out the unchanged amino-acids as the actual stimulants. It is quite conceivable that the qualitative make-up of the amino-acid fraction of a tissue bears some relation to its rate of heat production, quite aside from any change in the size of this fraction. In the postabsorptive period it seems reasonable to suppose that the proteins of the tissue and the free amino-acids and peptides held in it have reached, or approximated to some sort of an equilibrium, which is suddenly disturbed at times when there is an influx of amino-acids from the intestines, constituting a mixture quite different from that normal to the tissue. This disturbance in tissue equilibrium may possibly initiate chemical changes leading to an increased tissue metabolism until a readjustment is made as the resultant of catabolic and anabolic transformations, and after several hours an equilibrium is again established. The fact, as Lusk has shown, that the different amino-acids occurring in proteins have very different specific dynamic effects, perhaps lends some support to such theoretical considerations.

The revised conclusion of Lusk that the  $\alpha$ -hydroxyl- and  $\alpha$ -keto-acids arising in the metabolism of the amino-acids are the cell stimulants rather than the unchanged amino-acids themselves, does not remove all difficulties. In the first place there can be no assumption that these bodies accumulate in the tissues; in the absence of all direct evidence even that they exist in the tissues in any amount than traces. Nor is it plausible that they are formed only during protein digestion. The occurrence of the constant fraction of amino-acids in the tissues, even during prolonged fasting, probably indicates, as Van Slyke concludes, a continual building up and breaking down of protein material, with, therefore, a continual liberation of the  $\alpha$ -hydroxyl- and  $\alpha$ -keto-acids arising from the oxidative deamination of amino-acids. As far, therefore, as the quantitative occurrence in the tissues of unchanged amino-acids is concerned, on the one hand, and of the products of their deamination, on the other, there is little to choose in deciding which substances are responsible for the specific dynamic effect of protein. Against deciding in favor of the deaminized acids, it may be argued that the trend of

recent investigation has been to bring out the intimate relationship between the intermediary products of the metabolism of the simpler amino-acids and of glucose. For example, the large specific dynamic effect of alanine cannot with certainty be ascribed to the lactic acid supposed to arise from it in metabolism, since it is, perhaps, quite as probable that lactic acid is produced also in the normal metabolism of glucose, a substance possessing only a slight specific dynamic effect. Furthermore, hydroxyl- and keto-acids are produced in the metabolism of the fatty acids, and, while they are structurally different from those arising in the metabolism of amino-acids, it would be surprising if, in the one case, a large heat effect were produced in the tissues, while, in the other case, practically none at all results.

Against deciding upon the unchanged amino-acids as the stimulants to increased cellular metabolism, it has been argued by Lusk that Rubner, and later Hoobler, have shown that amino-acids, absorbed by the tissues and used in the formation of new tissue, have no specific dynamic effect. However, the work of Howland,\* in Lusk's laboratory, on the effect of casein on the heat production of a sleeping infant, may be interpreted as indicating a specific dynamic effect of the protein fraction retained for growth. In any case, the point at issue is of such importance and general interest as to require careful confirmation with the best and latest available methods. The peculiar sensitiveness of the infant to protein stimulation as compared with the adult, noticed by Howland, may perhaps be related to the fact established in this investigation that protein feeding in the young organism, as contrasted with the old, leads to a marked increase in the amino-acid concentration of the tissues.

#### CONCLUSIONS.

1. The concentration of amino-acids, ammonia, and urea in the tissues of rats is comparable to that of the tissues of other mammals thus far investigated.

2. In the young growing rat the concentration of amino-acid and of ammonia in the tissues is considerably higher than in the older animal.

\* Howland, J., *Z. physiol. Chem.*, 1911, lxxiv, 1.

3. In adult rats, protein feeding has only an inconsiderable effect upon the amino-acid concentration of the tissues, while increasing distinctly the urea content.

4. In young growing rats, on the contrary, protein feeding increases considerably the amino-acid and urea content of the tissues, and, less certainly, the ammonia content.

5. The ammonia and urea content of the livers of rats, both fasting and fed, is in general higher than that of the muscles.

This investigation was undertaken in cooperation with Mr. H. A. Shonle, who, however, withdrew from the University soon after the work was started. Acknowledgment is due to Mr. Shonle for valuable aid in deciding upon methods of procedure, in testing and revising such methods, and in performing some of the analytical work. The assistance of Mr. R. W. Dawley in the remainder of the analytical work is also appreciated. I also wish to acknowledge the interest and encouragement of Dr. H. S. Grindley, in whose laboratory the work was done.

## URINARY EXCRETION OF PHOSPHATES IN THE RABBIT.

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(Received for publication, October 12, 1918.)

The statement is commonly made that the phosphorus in the diet of carnivora is eliminated principally by the kidneys, while, in herbivora, phosphorus excretion takes place almost entirely through the intestine. Bischoff,<sup>1</sup> Müller,<sup>2</sup> Paton, Dunlop, and Aitchison,<sup>3</sup> Bergmann,<sup>4</sup> and others have shown that a large percentage of the phosphorus of the food intake is excreted by dogs in the urine. The urine of herbivora has, on the other hand, been stated by Bertram,<sup>5</sup> Jordan,<sup>6</sup> Tangl,<sup>7</sup> Emery and Kilgore,<sup>8</sup> Paton, Dunlop, and Aitchison,<sup>3</sup> and Bergmann,<sup>4</sup> to be practically free from phosphorus. The types of herbivora used in these studies were the ox, sheep, goat, and horse. The absence of phosphorus from the urine of herbivora is generally attributed to the high content of alkali compounds and calcium salts in the food, an alkaline urine containing carbonates having no solvent power for calcium phosphate. Weiske<sup>9</sup> found that, while on a vegetable diet only a trace of phosphorus was present in the urine of the goat, when fed on a diet of milk, this animal excreted over 20

<sup>1</sup> Bischoff, E., *Z. Biol.*, 1867, iii, 309.

<sup>2</sup> Müller, F., *Z. Biol.*, 1884, xx, 327.

<sup>3</sup> Paton, D. N., Dunlop, J. C., and Aitchison, R. S., *J. Physiol.*, 1899-1900, xxv, 212.

<sup>4</sup> Bergmann, W., *Arch. exp. Path. u. Pharmacol.*, 1901, xlvii, 77.

<sup>5</sup> Bertram, J., *Z. Biol.*, 1878, xiv, 335.

<sup>6</sup> Jordan, W. H., *Ann. Rep. Maine Agric. Exp. Station*, 1885-86, 42.

<sup>7</sup> Tangl, F., cited in *Jahresb. Fortsch. Thierchem.*, 1902, xxxi, 797.

<sup>8</sup> Emery, F. E., and Kilgore, B. W., *North Carolina Agric. Exp. Station, Bull.* 118, 1895, 245.

<sup>9</sup> Weiske-Proskau, H., *Z. Biol.*, 1872, viii, 246.

per cent of the food phosphorus in the urine. Paton, Dunlop, and Aitchison<sup>3</sup> reported that in dogs on a vegetable diet a large proportion of the phosphorus of the food was not excreted in the urine. Gouin and Andouard<sup>10</sup> showed that a calf receiving a diet of milk alone excreted 80 per cent of the food phosphorus in the urine and that phosphorus was deflected from the urine to the feces in proportion to the extent to which vegetable foods were substituted for milk in the diet.

The attention of the authors was attracted to the influence of diet on the urinary phosphorus excretion of rabbits during recent studies on alterations in the output of certain urinary constituents as determined by changes in the character of the diet.<sup>11</sup> It was noted that the proportion of the food phosphorus excreted in the urine varied widely according to the nature of the diet. Upon a diet of corn and oats the urinary output of phosphorus was far in excess of the intake of this element in the food. This excessive output of phosphoric acid in the urine when the animal was placed on a diet which contained a preponderance of acid-forming elements was regarded as a method of regulating acid-base equilibrium in the body of the rabbit. This is in accord with the work of Fitz, Alsberg, and Henderson,<sup>12</sup> who demonstrated by feeding hydrochloric acid to rabbits that the resultant shifting of the salt equilibrium toward the acid side caused an increased elimination of phosphates in the urine. The same effects were observed by the present authors in the studies cited above<sup>11</sup> after giving hydrochloric acid by stomach tube to rabbits.

As a result of these observations the series of experiments to be outlined in this paper was undertaken. The objects were to study further the relative proportions of phosphorus of the food intake excreted in the urine when the diet varied from acid-producing food, such as grain, to base-producing food, like carrots; to note the effect of subcutaneous injections of phosphates on the urinary phosphorus of the rabbit; and to observe the influence

<sup>10</sup> Gouin, A., and Andouard, P., *Soc. aliment. rationnn. bétail*, Compt. rend., 1907, 11th cong., 11-28, 127-130.

<sup>11</sup> Underhill, F. P., and Bogert, L. J., *J. Biol. Chem.*, 1916, xxvii, 161.

<sup>12</sup> Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113.

of diets consisting of acid-producing or base-producing foods and of injections of mono-, di-, and tribasic phosphates upon the relative proportion of injected phosphorus excreted in the urine.

### *Methods.*

Rabbits were confined in metabolism cages and the urine was collected in 24 hour periods by expressing it from the bladder by pressure through the abdominal wall. Each animal received either 100 gm. of oats, 500 gm. of carrots, or 50 gm. of oats and 250 gm. of carrots per day. The amounts actually eaten were recorded in the protocols. The phosphates in the urine were determined by titration with uranium acetate, while hydrogen ion concentration was estimated by the Henderson-Palmer method. Subcutaneous injections of solutions of the different sodium salts of phosphoric acid were given in turn at intervals of a few days and repeated with altered dietary conditions. The solutions of sodium phosphates were made up and standardized so that the 30 cc. of injected fluid contained, in each case, 150 mg. of phosphorus. There were no noticeable evil effects of the injections except local irritation caused by the injection of solutions of tribasic sodium phosphate. As there seemed to be a diminished urinary excretion of the injected phosphates after several injections had been given, a further series of experiments was carried out in which the rabbits received successive injections of the same phosphate solution ( $\text{Na}_2\text{HPO}_4$ ) while the diet remained constant. For the sake of brevity, specimen protocols only are given from each series of experiments.



*Rabbit 7, Male (Black); Weight 2,120 Gm. (Oct. 23).*

Date.	Urine vol- ume.	Specific gravity.	pH	Phos- phorus.	Notes.
1916	cc.			mg.	
Oct. 23	77	1.024	8.0	50	50 gm. oats, 228 gm. carrots.
" 24	172	1.013	7.48	79	50 " " 250 " "
" 25	76	1.038	9.27	48	50 " " 250 " "
" 26	96	1.028	8.7	167	50 " " 237 " "
					Injected (subcutaneous) 150 mg. P = 0.68 gm. $\text{Na}_2\text{HPO}_4$ in 30 cc. $\text{H}_2\text{O}$ .
Oct. 27	96	1.038	9.27	69	50 gm. oats, 250 gm. carrots.
" 28	120	1.028	8.7	87	50 " " 250 " "
" 29	130	1.014	8.7	63	50 " " 250 " "
" 30	208	1.017	8.7	145	50 " " 250 " "
" 31	150	1.016	7.48	198	50 " " 250 " "
					Injected 150 mg. P = 0.79 gm. $\text{Na}_2\text{PO}_4$ in 30 cc. $\text{H}_2\text{O}$ .
Nov. 1	190	1.016	8.7	125	50 gm. oats, 250 gm. carrots.
" 2	114	1.017	8.7	100	50 " " 250 " "
" 3	180	1.021	8.7	51	50 " " 250 " "
" 4	164	1.015	8.0	46	50 " " 250 " "
" 5	163	1.017	8.0	86	50 " " 250 " "
" 6	152	1.017	6.9	150	50 " " 250 " "
					Injected 150 mg. P = 0.58 gm. $\text{NaH}_2\text{PO}_4$ in 30.7 cc. $\text{H}_2\text{O}$ .
Nov. 7	152	1.016	7.48	87	50 gm. oats, 250 gm. carrots.
" 8	166	1.017	8.7	87	50 " " 250 " "
" 9	305	1.011	8.0	65	500 " "
" 10	380	1.012	8.7	138	Wt. 2,100 gm. 500 " "
" 11	335	1.010	8.0	97	500 " "
" 12	427	1.013	8.7	114	500 " "
" 13	385	1.011	8.7	107	500 " "
" 14	280	1.015	7.48	117	500 " "
					Injected 150 mg. P = 0.68 gm. $\text{Na}_2\text{HPO}_4$ in 30 cc. $\text{H}_2\text{O}$ .
Nov. 15	460	1.011	8.0	97	500 gm. carrots.
" 16	340	1.011	8.0	66	500 " "
" 17	355	1.011	8.7	79	500 " "
" 18	420	1.011	8.7	81	500 " "
" 19	320	1.010	8.7	101	500 " "
" 20	360	1.013	7.48	135	500 " "
					Injected 150 mg. P = 0.58 gm. $\text{NaH}_2\text{PO}_4$ in 30.7 cc. $\text{H}_2\text{O}$ .

*Rabbit 7—Concluded.*

Date.	Urine vol- ume.	Specific gravity.	pH	Phos- phorus.	Notes.
<i>1916</i>	<i>cc.</i>			<i>mg.</i>	
Nov. 21	430	1.011	8.7	73	500 gm. carrots.
" 22	290	1.016	8.7	51	500 " "
" 23	383	1.014	8.7	127	500 " "
					Injected 150 mg. P = 0.79 gm. $\text{Na}_3\text{PO}_4$ in 30 cc. $\text{H}_2\text{O}$ .
Nov. 24	350	1.016	8.7	66	500 gm. carrots.
" 25	440	1.014	8.7	61	500 " "
" 26	318	1.017	8.7	61	500 " "
" 27	58	1.034	8.7	72	Wt. 2,060 gm. 100 gm. oats.
" 28	31	1.044	6.9	76	100 gm. oats.
" 29	21	1.060	6.0	72	100 " "
" 30	30	1.052	5.7	104	75 " "
Dec. 1	37	1.041	5.3	149	32 " "
					Injected 150 mg. P = 0.68 gm. $\text{Na}_2\text{HPO}_4$ in 30 cc. $\text{H}_2\text{O}$ .
Dec. 2	29	1.052	5.7	108	48 gm. oats.
" 3	33	1.046	5.3	107	57 " "
" 4	31	1.042	5.3	90	75 " "
" 5	71	1.017	5.3	107	Wt. 2,140 gm. 22 gm. oats.
					Injected 150 mg. P = 0.58 gm. $\text{NaH}_2\text{PO}_4$ in 30.7 cc. $\text{H}_2\text{O}$ .
Dec. 6	30	1.036	5.3	85	31 gm. oats.
" 7	40	1.033	5.3	95	10 " "
" 8	110	1.017	5.3	182	Refused food. Injected 150 mg. P = 0.79 gm. $\text{Na}_3\text{PO}_4$ in 30 cc. $\text{H}_2\text{O}$ .
Dec. 9	40	1.030	5.3	90	Refused food.
" 10	87	1.025	5.3	156	" " Albuminuria.
" 11					Returned to pen, condition fair. Fed car- rots, condition good.

*Rabbit 12, Female (Brown, Pregnant); Weight 2,840 Gm. (Dec. 11).*

Date.	Urine vol- ume.	Specific gravity.	pH	Phos- phorus.	Notes.
1916	cc.			mg.	
Dec. 11					415 gm. carrots.
" 12	255	1.013	8.0	50	352 " "
" 13	158	1.018	8.0	77	345 " "
" 14	305	1.015	8.0	75	500 " "
" 15	110	1.025	7.48	184	230 " "
					Injected 150 mg. P = 0.68 gm. $\text{Na}_2\text{HPO}_4$ in 30 cc. $\text{H}_2\text{O}$ .
Dec. 16	81	1.035	8.7	85	324 gm. carrots.
" 17	234	1.015	8.7	54	403 " "
" 18	90	1.031	7.48	173	228 " "
					Injected 150 mg. P = 0.68 gm. $\text{Na}_2\text{HPO}_4$ .
Dec. 19	100	1.036	9.27	62	390 gm. carrots.
" 20	196	1.028	9.27	66	362 " "
" 21	150	1.023	9.27	161	370 " "
					Injected 150 mg. P = 0.68 gm. $\text{Na}_2\text{HPO}_4$ .
Dec. 22	174	1.021	9.27	60	445 gm. carrots.
" 23	234	1.020	9.27	80	457 " "
" 24	222	1.019	9.27	140	474 " "
					Injected 150 mg. P = 0.68 gm. $\text{Na}_2\text{HPO}_4$ .
Dec. 25	207	1.023	9.27	87	495 gm. carrots.
" 26					Returned to pen, condition good. Wt., 2,880 gm.

#### DISCUSSION.

Upon inspection of the protocols and tables, several facts of interest are to be noted. First, there is a greater excretion of phosphorus by the urine in the case of the rabbit than in other herbivora previously studied and secondly, this urinary phosphorus may be made to vary largely by changes in the diet. Thus rabbits, even on a diet composed exclusively of carrots, where a strongly alkaline urine is secreted, eliminate about 25 per cent of the food phosphorus by way of the kidneys, while on a diet of oats more than 100 per cent of the food phosphorus is

excreted in the urine. When a mixed diet of oats and carrots is fed, the urine contains about 50 per cent of the phosphorus ingested in the food. The urine of the animals consuming both the carrot and mixed diets is alkaline, but that of the rabbits fed on the grain diet is acid. Moreover, the calcium content of the food is highest in the carrot diet and lowest in the grain diet. Doubtless both the reaction of the urine and the calcium content of the diet are factors in effecting alterations in urinary phosphate excretion by changes in the character of the diet. Difference in utilization of the various diets is another possible factor to be taken into consideration. A summary of these results is found in Table I.

TABLE I.

*Effect of Alterations in the Character of the Diet upon the Urinary Excretion of the Ingested Phosphorus.*

Rabbit No.	P intake per day.			Average urinary P per day.			P excreted in urine per day.		
	Mixed diet.	Carrot diet.	Grain diet.	Mixed diet.	Carrot diet.	Grain diet.	Mixed diet.	Carrot diet.	Grain diet.
	mg.	mg.	mg.	mg.	mg.	mg.	per cent	per cent	per cent
4	165	240	90	87	59	112	52	24	124
5	165	240	90	50	62	89	30	26	99
6	165	240	90	101	65	110	61	27	122
7	165	240	90	89	63	108	53	26	120
Average.....				82	62	105	49	26	116

In respect to the fate of injected phosphates, there has been a fairly general agreement that such phosphates were excreted by carnivora chiefly in the urine and by herbivora almost entirely in the feces. Falek<sup>13</sup> found that sodium phosphate injected intravenously into dogs was quickly eliminated by the kidneys. The same result was obtained by Bergmann<sup>4</sup> after injecting dogs subcutaneously with sodium phosphate. He reported, however, that the urinary phosphorus of a wether remained unweighable after similar injections of phosphate. Paton, Dunlop, and Aitchison<sup>3</sup> state that, in the goat, none of the phosphoric acid subcutaneously injected as sodium phosphate is excreted in the urine while, in

<sup>13</sup> Falek, C. P., *Virchows Arch. path. Anat.*, 1872, liv, 173.

the dog as well, a large proportion of such injected phosphate is not excreted in the urine. Mendel and Underhill<sup>14</sup> injected both sodium phytate and disodium acid phosphate subcutaneously during a series of balance experiments on a dog with the result that the excess of phosphorus was eliminated almost entirely through the kidneys rather than in the feces. After similar injections in a goat, they found no increase in the phosphates of the urine.

The present series of experiments shows that the rabbit, in contrast to such other herbivora as the goat, excretes an excess of phosphates to a large extent through the kidneys. While there were considerable variations in the percentage of injected phosphates which appeared in the urine, injections of any one of the

TABLE II.

*Effect of Successive Injections of Phosphate on the Urinary Excretion of Injected Phosphorus.*

Rabbit No.	Diet.	Substance injected.	Injected P excreted in urine. Injection No.			
			1	2	3	4
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
12	Carrot.	$\text{Na}_2\text{HPO}_4$	73	78	63	40
14	"	$\text{Na}_2\text{HPO}_4$	36	47	30	0
18	Mixed.	$\text{Na}_2\text{HPO}_4$	89	80	80	61

three phosphates of sodium invariably caused a distinct rise in urinary phosphates, a rise frequently equivalent to as much as 70 to 100 per cent of the injected phosphorus. So far as we were able to observe, the nature of the phosphate injected or of the diet consumed had no constant effect on the amount of injected phosphorus excreted in the urine. Such observations were complicated by variations in phosphate elimination in individual rabbits and by the effect of successive injections of phosphates. These successive phosphate injections seem either to lessen the ability of the animal to excrete the injected phosphorus through the kidney, to develop an increased excretion of phosphate by the intestine, or to lead to phosphorus storage in the organism. The fact that, after successive subcutaneous injections of sodium

<sup>14</sup>Mendel, L. B., and Underhill, F. P., *Am. J. Physiol.*, 1906-07, xvii, 75.

phosphates, less of the injected phosphorus is excreted in the urine than after the first injection is shown by the experiments on Rabbits 12, 14, and 18, summarized in Table II. Table III shows the percentage of injected phosphorus excreted through the kidneys by various rabbits after initial injections of phosphate, the nature of the diet and of the phosphate injected being altered in different cases.

The effect of subcutaneous injections of phosphates on the hydrogen ion concentration of the urine was not pronounced.

TABLE III.

*Urinary Excretion of Injected Phosphorus after Initial Injections of Phosphates with Varying Dietary Conditions.*

Rabbit No.	Diet.	Substance injected.	Injected P excreted in urine.
			<i>per cent</i>
4	Mixed.	$\text{Na}_2\text{HPO}_4$	96
5	"	$\text{Na}_2\text{HPO}_4$	29
6	"	$\text{Na}_2\text{HPO}_4$	80
7	"	$\text{Na}_2\text{HPO}_4$	79
16	"	$\text{Na}_2\text{HPO}_4$	105
18	"	$\text{Na}_2\text{HPO}_4$	89
19	"	$\text{Na}_2\text{HPO}_4$	55
12	Carrot.	$\text{Na}_2\text{HPO}_4$	73
13	"	$\text{Na}_2\text{HPO}_4$	75
14	"	$\text{Na}_2\text{HPO}_4$	35
8	Grain.	$\text{Na}_2\text{HPO}_4$	80
9	"	$\text{Na}_2\text{HPO}_4$	50
10	"	$\text{Na}_3\text{PO}_4$	67
11	"	$\text{NaH}_2\text{PO}_4$	73
			Average 76
			Average 61
			Average 67

The increased content of the urine in phosphates following such injections was usually accompanied by a slight rise in hydrogen ion concentration but, as might be expected in such a mixture of buffer substances, relatively enormous increases of phosphate were observed with but slight alteration in the reaction of the urine.

## SUMMARY.

Rabbits, in contrast to most other herbivora, excrete a considerable amount of phosphates through the kidneys.

The proportion\* of food phosphorus eliminated in the urine by rabbits may be caused to vary widely by changes in the nature of the diet, being about 25 per cent on a carrot diet, 50 per cent on a mixed diet of carrots and oats, and over 100 per cent on a diet composed exclusively of oats.

Solutions of mono-, di-, and trisodium phosphates injected subcutaneously each caused a considerable increase in urinary phosphates, 70 to 100 per cent of the injected phosphorus having frequently appeared in the urine.

After successive subcutaneous injections of phosphate, less of the injected phosphorus is excreted in the urine than after the initial injection.

The character of the diet and the nature of the phosphate injected had no apparent influence on the percentage of injected phosphorus excreted by the kidneys. It is impossible, however, to draw general conclusions with regard to these factors on the basis of these experiments, due to variations in urinary phosphorus excretion in individual rabbits and to the effect of repeated injections of phosphate solutions on the urinary elimination of phosphates subsequently injected.

Large increases in the phosphate content of the urine were noted, accompanied by only slight increases in hydrogen ion concentration.

# **STUDIES IN THE METABOLIC CHANGES INDUCED BY THE ADMINISTRATION OF GUANIDINE BASES.**

## **V. THE CHANGE OF PHOSPHATE AND CALCIUM CONTENT IN SERUM IN GUANIDINE TETANY\* AND THE RELATION BETWEEN THE CALCIUM CONTENT AND SUGAR IN THE BLOOD.**

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(Received for publication, July 27, 1918.)

In a recent communication (1, 2) it was demonstrated that a marked acidosis appears in the condition of guanidine tetany. This same phenomenon is present in parathyroid tetany and in idiopathic tetany (3) in both of which conditions the same evidences are observed in the urine, such as increased elimination of  $\text{NH}_3$ , decreased acid excretion, and the lowering of the hydrogen ion concentration. Greenwald (4, 5, 6) found that in parathyroid tetany in dogs the elimination of phosphate was distinctly diminished and that this diminution was accompanied by a marked retention of phosphate in the blood. We suggested that the phosphate retained served to neutralize the acid probably produced by the muscular activity caused by the increased content of guanidine base present when the parathyroid is removed (7, 8, 9).

In connection with the study of the cause of tetany, several investigators estimated the change of salt excretion, especially calcium, in the urine and feces, and the content in the tissues, especially the brain. Among these workers Sabbatani (10), first suggested that the decreased content of calcium in the brain caused the irritability of the nervous tissue, since he had observed that when calcium chloride was applied to the cortical surface the irritability was immediately reduced. With antagonistic agents, such as sodium citrate, the reverse is true. He thought this might be a factor contributing to the cause of convulsive diseases. Following the above suggestion Quest (11) first found that the calcium content of the brains of patients dying in tetany was distinctly diminished when compared to the normal brain of the same age. There was also an increased ratio of nitrogen to calcium. Later workers, including Aschenheim (12) in idiopathic tetany,

\* The preliminary work on this subject was reported in the Proceedings of the Society for Experimental Biology and Medicine for May 15, 1918.



and MacCallum and Voegtlin (13), Pexa (14), Aschenheim (12), Weigert (15), in tetania parathyreopriva also state that the content of calcium in the brain is diminished. On the other hand Stoeltzner (16), Leopold and von Reuss (17), and Cohn (18), among others, claim that the calcium content of the brain is approximately normal in idiopathic tetany. Cooke (19) likewise reported that the calcium content of the brain in two dogs in experimental tetany, did not change or was rather slightly higher than in control animals. The magnesium was also practically normal.

Comparing the normal arterial blood in dogs with that after parathyroidectomy, MacCallum and Voegtlin (13), found a marked decrease in calcium and they state that this decrease in the blood and tissues is caused by the increased excretion in the urine and feces. Neurath (20) also demonstrated the diminution of precipitable calcium in the blood.

Beside the work of MacCallum and Voegtlin on the calcium excretion cited above, Cooke (19) claims that the elimination of calcium in the urine is almost unchanged but is increased in the feces. The magnesium excretion is markedly increased in the urine. Recently Kojima (21) stated in his metabolism experiment in rats that after parathyroidectomy there seems to be an increase of calcium in the urine and less retained in the body. Von Cybulski (22) and Schwarz and Bass (23), working with patients suffering from idiopathic tetany in feeding experiments, state that upon the appearance of the tetanic symptoms the calcium retention is lowered. We must also call attention to the work of Iselin (24), Hohlbaum (25), Toyofuku (26), and Morel (27) in which they demonstrated a delayed bone growth, delayed healing of fractures, and insufficient calcification of the teeth in parathyroidectomized rats. The experiments cited above suggest either that the calcium present is changed in some way or that there is an actual change in quantity. Paton and Findlay (28) expressed the opinion that a decreased calcium content of the tissues and an increased elimination of calcium is not a satisfactory explanation although administration of calcium strikingly diminished the electrical excitability and caused a disappearance of the symptoms in parathyroid tetany.

After perusal of the abundant literature concerning this problem, it appears that in parathyroid tetany or in idiopathic tetany the excretion of calcium may be increased in most cases and that at the same time the calcium may be decreased in the blood although all of this evidence is not certain. In the first paper of this series, we mentioned that the true cause of tetany was some toxin, such as guanidine bases, circulating in the blood, which was produced in some way by the lack of the parathyroids. The injection of such guanidine base as guanidine hydrochloride into the rabbit, showed almost the same metabolic evidences in the urine and blood, accompanied by acidosis, which is considered by Morel (29) and some other workers to be the fun-

damental cause of tetany. Therefore, we may suppose that this condition of acidosis may influence the acid-base equilibrium, and it is necessary to estimate the phosphate and calcium content of the blood in guanidine tetany to compare with that in parathyroid tetany.

### *Methods.*

Rabbits were used in this investigation. The content of phosphate and calcium was estimated before and after the administration of guanidine hydrochloride to compare the normal and tetanic condition. About 7 cc. of blood were drawn from the jugular vein and 1 cc. was used for the sugar estimation. The rest of the blood was kept over night for the separation of the serum. Marriott's (30, 31) methods for phosphate and calcium were employed, and his technique was followed.

In some cases after the administration of guanidine, the fat content was, apparently, very much increased. On filtering the calcium oxalate or ammonium magnesium phosphate through a Gooch crucible and washing with dilute ammonia, a colloidal solution is sometimes obtained which is very difficult to filter. This can be overcome by adding a small quantity of alcoholic ammonia to the crucible. This procedure results in no loss of phosphate or calcium. It is necessary to maintain uniform conditions of the animals in estimating the phosphate content of the normal rabbits. Oats and water only were given to the first group of rabbits, and oats, greens, and water to the second group throughout the investigation. In the first group the first blood was taken after the rabbits had been kept in the cage 24 hours, and after another 24 hours the injection of guanidine hydrochloride was performed. The second sample of blood must be taken after the acidosis becomes severe; but half of the rabbits of this group died before the blood was drawn. The data mentioned in Table I were obtained from samples taken from about 24 to 48 hours after administering the drug. A long continued acidosis was intended to be maintained in the second group of animals and for this purpose several small doses of guanidine were administered at about 1 day intervals and the rabbits were fed only greens and water after the injections.

In thirty-three estimates on twenty-eight rabbits, the normal content of phosphate varies from 2.1 to 5.6 mg. per 100 cc. of serum. This is a rather large variation when compared to the calcium content which varies from 10.5 to 13.0 mg. per 100 cc. of serum. The phosphate content may vary with the condition of the animal, the food, or with the environment. It may be parallel to the hydrogen ion concentration of the blood. The rabbits which had albumin in the urine show a rather high content of phosphate in the serum; Rabbit 4, which had heavy albuminuria and was pregnant, showed a high content; No. 5 also had heavy albuminuria and a high content of phosphate in the serum. Rabbit 1 was examined on three different days. The first estimate was 3.5 mg., but the third was the highest normal figure obtained—5.6 mg. This shows how variable is the normal figure for phosphate in the blood although this animal had albumin in the urine. We may consider that the normal content of phosphate in normal rabbits is from 2 to 4 mg. per 100 cc. of serum and that when above 4 mg. we may consider we have an acidosis. None of the rabbits employed in this investigation had sugar in the urine.

After the injection of guanidine almost all animals showed an increased content of phosphate in the blood and in some cases, Nos. 14, 22, 2, and 3, the amount was increased to five times the normal figure. Rabbit 14 was in moribund condition when the sample of blood was taken, 51 hours after guanidine injection; the same was true of No. 22, 25 hours after injection; No. 2, 22 hours after injection; No. 3, 15 hours after injection. All of these four rabbits showed increases about five times the normal. From these experiments the fact is established that in guanidine tetany the phosphate content of the blood is in almost all cases increased except Nos. 6, 12, and 5, but even here the values kept above what we consider the highest normal limits.

Another important evidence is the decreased calcium content, though it is not so marked. In Table I we have arranged in descending magnitude the phosphate content after guanidine injection. It may be noted that in all cases in which the phosphate reaches 6 mg. in 100 cc. of serum there is a decrease in calcium except No. 13 in which the phosphate rose so high that there was practically no change in calcium. The second speci-

TABLE I

*Phosphate and Calcium Content in Serum before and after the Administration of Guanidine Hydrochloride.*

Rabbit No.	Albumin in urine.	Guanidine per kg. of body weight.	Phosphate and calcium content, and sugar before and after injection of guanidine.					
			Before.			After.		
			Phosphate.	Calcium.	Sugar.	Phosphate.	Calcium.	Sugar.
		gm.	mg. per 100 cc.	mg. per 100 cc.	per cent	mg. per 100 cc.	mg. per 100 cc.	per cent
14	—	0.27	4.0	12.0	0.133	18.2	8.7	0.072
22	—	0.15	3.0	12.6	0.133	16.0	10.0	
2	—	0.25	2.1	12.0	0.105	11.3	8.5	0.071
3	—	0.20	2.1	11.1	0.120	10.7	8.8	0.051
13	—	0.20	3.6	11.0	0.149	9.8	12.6	0.059
15	—	0.30	4.0	13.0	0.100	9.0	8.6	0.053
9	—	0.27	2.8	12.0	0.100	8.6	10.4	0.056
19	—	0.25	2.1	11.8	0.120	7.8	10.4	0.047
11	++	0.30				7.4	10.2	0.043
10	+	0.30				6.8	10.2	0.055
4	+++	0.20	4.0	11.0	0.087	6.0	11.2	0.085
27	—	0.15	2.4	12.4	0.125	6.0	11.0	0.053
7	—	0.30	3.8	11.0	0.111	5.4	12.0	0.083
6	++		4.0	12.4	0.143			
6	++	0.30	4.6	12.6	0.100	4.6	12.6	0.100
12	++	0.30	3.4	12.6		4.3	12.0	0.059
5	+++	0.33	4.5	10.5	0.091	4.0	11.0	0.084
1	++		3.5	11.1	0.111			
1	++		5.1	11.5	0.125			
1	++	0.20	5.6	10.8	0.125			
8	+	0.20	4.8	12.0	0.130			
17	—	0.20	2.8	12.8	0.109			
16	++	0.20	3.0	12.7	0.111			
18	++	0.20	3.0	12.4	0.125			
20	+	0.15	4.2	12.6	0.107			
21			3.2	11.0	0.143			
30	—	0.15	3.4	12.2	0.130			

men of blood was drawn 26 hours after injection and the rabbits manifested distinct tetanic symptoms and also were much more sensitive than the other animals. The phosphate increased but was not followed by the decrease in calcium. In general the increased phosphate is accompanied by a decrease in calcium and

it is clear that the rise in phosphate is followed by the fall in calcium from the fact that a rise in phosphate has been observed while the calcium remains normal. However, a fall in calcium with a normal phosphate has never been observed. More will be said of this in the discussion of the second group of rabbits.

In Table II is shown the ratio of phosphate and calcium before and after administration of guanidine injection. In Rabbit 2 the increase in phosphate over the normal before injection was 538 per cent, and in all other rabbits except Nos. 5 and 6, there was a distinct increase. The calcium in some cases decreased to 71 per cent of its initial values, and the others all decreased with the exception of a few cases. In normal conditions the ratio of calcium to phosphate varies from 5.7 to 1.9, but after guanidine injection it decreases to 2.8 to 0.5. If we exclude the "normal" rabbits showing albuminuria, the normal value of  $\frac{\text{calcium}}{\text{phosphate}}$  is 3.0 or over. Therefore, we may say that in almost all cases after guanidine injection the ratio  $\frac{\text{calcium}}{\text{phosphate}}$  decreases.

In the second group of rabbits we intended to secure more definite information on the decreased calcium content of blood following increased phosphate content. It was necessary to keep animals for a longer time, and for this rather small doses of guanidine were given occasionally and blood specimens were drawn at intervals. Nine rabbits were tried in this group, and in only a few cases were satisfactory results obtained. In Rabbit 23 (Table III) the normal phosphate content was 3.2 mg. and the calcium 12.1 mg. per 100 cc. of serum. After several injections of the drug, the phosphate rose to 5.2 and the calcium decreased to 6.6, about half of its normal value. The small injections of drug were continued and in the last specimen from this rabbit the phosphate decreased to 4.6 and the calcium was again restored toward normal though not reaching the true normal level. This shows that as the acidosis subsides the calcium is restored. Rabbit 24 shows almost the same phenomena though the decrease in calcium is not so marked as in the above case. In this rabbit the last specimen of blood contained less phosphate than former specimens but the calcium was not restored to normal; rather it decreased

TABLE II.

*Relation between the Phosphate and Calcium Contents before and after Injection of Guanidine and the Ratio of Calcium to Phosphate before and after Injection of Guanidine.*

abbit No.	Ratio of phosphate and calcium contents after, to the phosphate and calcium contents before injection of guanidine.		The ratio of calcium to phosphate before and after injection of guanidine.	
	Phosphate (after) Phosphate (before)	Calcium (after) Calcium (before)	Calcium Phosphate (before).	Calcium Phosphate (after).
14	4.55	72	3.0	0.5
22	5.33	79	4.2	0.6
2	5.38	71	5.7	0.7
3	5.10	79	5.3	0.8
13	2.72	114	3.1	1.3
15	2.25	66	3.2	1.0
89	3.07	87	4.3	1.2
19	3.71	88	5.6	1.3
11				1.4
10				1.5
4	1.50	102	2.8	1.9
27	2.50	89	5.2	1.8
7	1.44	109	2.9	2.2
6			3.1	
6	1.00	100	2.7	2.7
12	1.26	96	3.7	2.8
5	0.89	105	2.3	2.8
1			3.2	
1			2.3	
1			1.9	
8			2.5	
17			4.1	
16			4.2	
18			4.1	
20			3.0	
21			3.4	
30			3.6	

slightly. This shows that phosphate and calcium do not change distinctly parallel and supports the view that the change of phosphate is the preliminary change and that the calcium decrease or restoration is a secondary phenomenon following acidosis. The results of Rabbit 25 substantiate this theory; the first specimen of blood after injection of the drug showed an increase in phosphate

TABLE III.

*Phosphate and Calcium Content in Serum before and after the Administration of Guanidine Hydrochloride.*

Rabbit No.	Day and time.	Guanidine per kg. of body weight.	Phosphate and calcium content before and after injection of guanidine.		Sugar.	Remarks.
			Phosphate.	Calcium.		
		gm.	mg. per 100 cc.	mg. per 100 cc.	per cent	
23	1st, 3 p.m.		3.2	12.1	0.125	Wt., 1,660 gm.; moderate albumin but no sugar in urine.
	2nd, 3 "	0.1				
	3rd, 2 "	0.05				
	4th, 8 a.m.	0.05				
	1 p.m.		5.2	6.6	0.123	
	5 "	0.05				Animal killed.
	5th, 8 a.m.	0.05				
	2 p.m.	0.05				
	3 "		4.6	9.5		
24	1st, 4 p.m.		3.8	12.0	0.120	Wt., 1,460 gm.; very faint trace of albumin but no sugar in urine.
	2nd, 3 "	0.1				
	4th, 2 "	0.05				
	5th, 8 a.m.	0.05				
	2 p.m.		5.8	10.0	0.105	
	5 "	0.05				Animal killed.
	6th, 8 a.m.	0.05				
	2 p.m.	0.05				
	7 "		4.4	9.7	0.100	
25	1st, 2 p.m.		2.9	11.6	0.109	Wt., 2,520 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	4th, 5 p.m.	0.08				
	5th, 5 "	0.03				
	6th, 11 a.m.		4.9	11.2	0.091	
	5 p.m.	0.07				Moribund; killed.
	7th, 2 "				0.122	
	8th, 10 a.m.	0.10				
	9 p.m.		2.8	10.0	0.120	
26	1st, 3 p.m.		3.2	12.3	0.137	Wt., 2,220 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	4th, 5 p.m.	0.08				
	5th, 5 "	0.03				
	6th, 11 a.m.		4.8	12.0	0.125	
	5 p.m.	0.07				Animal killed.
	7th, 3 "		6.8	6.0	0.143	

TABLE III—*Concluded.*

Rabbit No.	Day and time.	Guanidine per kg. of body weight.	Phosphate and calcium content before and after injection of guanidine.		Sugar.	Remarks.
			Phosphate.	Calcium.		
		gm.	mg. per 100 cc.	mg. per 100 cc.	per cent	
28	1st, 4 p.m.		2.2	12.2	0.112	Wt., 2,920 gm.; trace of albumin but no sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 2 p.m.	0.08				
	6th, 11 a.m.		4.4	11.0	0.137	
	4 p.m.	0.07				
	7th, 10 a.m.	0.07				Died 2 hours after last sample.
	8th, 11 "		4.0	9.7	0.115	
	4 p.m.	0.10				
	9th, 9 a.m.	0.05				
	2 p.m.		4.0	10.5	0.100	
29	1st, 3 p.m.		3.6	12.0	0.125	Wt., 1,990 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 4 p.m.	0.08				
	6th, 4 "		3.0	10.2	0.122	
	5 "	0.07				
	7th, 4 "	0.10				Found dead 10th day, 8 a.m.
	8th, 9 a.m.	0.07				
	3 p.m.		4.3	11.0	0.117	
	9th, 3 "	0.10				
31	1st,* 2 p.m.		4.0	11.4	0.111	Wt., 1,480 gm.; trace of albumin but no sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 10 "	0.10				Killed.
	6th, 12 m.		5.8	10.0	0.102	
32	1st, 3 p.m.		3.6	11.6	0.125	Wt., 2,150 gm.; no albumin or sugar in urine.
	3rd, 9 a.m.	0.15				
	5th, 10 "	0.10				
	6th, 3 p.m.		3.5	10.0	0.120	
	4 "	0.08				
	7th, 9 a.m.	0.07				Killed.
	4 p.m.		4.2	12.0	0.105	
	8th, 11 a.m.		6.0	10.4	0.120	
21	1st, 4 p.m.		6.2	8.0	0.139	Wt., 2,040 gm.; trace of albumin in urine.
	3rd, 9 a.m.					
	10 "		6.4	7.7	0.270	Died with convulsion.



from 2.9 to 4.9, but calcium still remained normal. In the second specimen after injection, the phosphate decreased but calcium was less than in the former specimen. Rabbit 26 distinctly substantiates the above theory; the first specimen after guanidine administration shows a rise in phosphate from 3.2 to 4.8, and calcium is unchanged; in the last specimen phosphate rises to 6.8 mg., and the calcium decreases to 6.0, about half the content of the normal. Rabbits 28 and 29 also show a similar behavior though not so distinct.

Rabbit 21 of this group had spontaneous acidosis. This rabbit was very sensitive and excitable. The blood before injection of guanidine contained 6.2 mg. of phosphate per 100 cc. of serum, which is much higher than in normal rabbits, and the calcium was 8.0 mg. After a small dose of guanidine the animal showed a more severe dyspnea than usual. Such a small dose—1.2 mg. per kilo of body weight—is not enough to induce such severe symptoms in normal rabbits. The rabbit died in convulsions 1 hour after the injection. The blood drawn at death contained 6.4 mg. of phosphate and 7.7 mg. of calcium, which are practically the same values as those before injection of guanidine in this rabbit, and so we may say that the drug had no influence on the calcium and phosphate in this short time. These facts strengthen the view that the acidosis plays an important part in the reduction of calcium in the blood.

#### DISCUSSION.

From the results of the present experiments, and of previous papers on guanidine tetany it is plain that following muscular activity a severe acidosis appears and that phosphate is retained in the body.

In the present experiment the acidosis became severe and the phosphate reaches a value above 6.0 mg. and is sustained, while the calcium may be decreased in these cases of guanidine tetany.

In the abundant literature on calcium in tetania parathyreopriva and idiopathic tetany some writers report a distinct decrease of calcium in the tissues and blood together with an increased excretion, while other authors found no appreciable change from the normal. Likewise in our experiments on guanidine tetany some cases show a distinct decrease in calcium while some remain

normal. We may conclude that in parathyroid tetany the decrease of calcium is not constant, and we may also suggest that the calcium decrease is not the fundamental cause of tetany, but it may be a secondary phenomenon of altered metabolism. Here we may call attention to the work of MacCallum and Voegtlin (13) who state:

"It was shown that the transfusion of blood from a normal dog will suppress the symptoms of tetany when a sufficiently large amount is introduced. Emulsion of brain substance was injected in the same way but the effect upon the tetany was so indefinite that it was questioned in that paper whether the action of the emulsion was any more marked than would have been that of so much salt solution or emulsion of any other organ from a normal dog. Emulsions of the parathyroid gland seemed to have a definitely specific effect but that appears only after the lapse of some hours whereas the effect of bleeding and infusion of relatively large amounts of salt solution or blood (200 to 400 cc.) from a normal dog has an almost immediate effect in suppressing the tetany. We could find no other explanation of this phenomenon than that some poisonous material, not destroyed or present in larger amounts than physiologically, on account of the absence of the parathyroid glands, was circulating in the blood."

In accord with the toxin theory, the increased content of guanidine was recently found. After injecting this toxin into animals they manifest almost the symptoms of tetany and also physiological and metabolic evidences, as is shown in the previous work and in the present experiment. We may now conclude that the true cause of tetany is the increased content of guanidine resulting from a disturbance in the function of the parathyroid. Another question naturally arises: How does the calcium injection temporarily abolish the tetany? We may imagine that in tetany the calcium of the tissues and blood may be inactivated or precipitated and excreted and that when soluble calcium is replaced or supplied to the normal content, the excitability of the nerve is reduced. Beside the work of Sabbatani (10), that of Loeb (32), Friedenthal (33), and also Garrey (34) in which is shown that when skeletal muscles are immersed in solutions which precipitate calcium less electrical stimulus is required to cause a contraction, supports the above view. MacCallum (35) also reported that when certain salts which precipitate calcium are administered the peristalsis of the intestine is increased. This also supports the view that the calcium content of the brain is instru-

mental in the excitability of the muscles. Starkenstein (36) reported that the introduction of phosphate and citrate in the form of sodium salts provokes muscular twitching. The fact is that the salts mentioned are precipitants of calcium. Quest (37) and many others made the interesting observation in feeding experiments that animals fed with calcium-free food or a diet with diminished calcium, showed an electrical hyperexcitability.

Recently MacCallum, Lambert, and Vogel (38) reported that in perfusion experiments in which dialyzed blood poor in calcium was perfused through the isolated extremity an extreme hyperexcitability was produced, quite like that observed in tetany. When the isolated limb was perfused with normal blood there was no hyperexcitability produced. They also established the fact that when tetanic animals were bled, and the blood was replaced by normal blood, the tetany was relieved and the hyperexcitability was lowered, though when dialyzed blood poor in calcium was replaced there were practically no changes. From this they support the deficiency of calcium theory of tetany. From the above works we may judge that in tetany the diminished calcium content of the brain is accountable for part of the hyperexcitability of the nerves, and replacement of the soluble calcium only diminishes part of the excitability in tetany. In other convulsive diseases, such as eclampsia and uremia, the calcium excretion is increased and the calcium content of the brain diminished (11). In tetany, however, the mechanism for the diminution of calcium in the tissues may be attributed to the acidosis since acid administration to animals causes an increased excretion of calcium (39) and magnesium, though this has not been so definite (40) until the present time.

*Relation between Sugar and Calcium Content in the Blood in Guanidine Tetany.*

In a preceding paper (41) we demonstrated that in guanidine tetany the content of sugar in the blood was slightly increased for the first few hours, especially after a large dose of the drug. This increase comes from the developing dyspnea caused by the disturbance of the respiratory center by the increased hydrogen ion concentration incident to the developing acidosis. On the contrary, several hours after the injection of the drug there appears a

hypoglycemia, while in some cases the blood sugar falls to half the normal quantity. In the present investigation we estimated the sugar in the blood simultaneously with the phosphate and calcium. The marked hypoglycemia was also produced in the first group of rabbits, half of them showing a decrease of half the normal. Comparing the calcium and sugar content in the blood of the same animals, it is clear that there is no relation between these two components of the blood. In Nos. 13, 12, and 27 the sugar decreases to half of the normal value while the calcium maintains the normal level.

Underhill and Blatherwick (42, 43) found that in parathyroid tetany the sugar content is markedly decreased and that the normal level can be restored by the injection of soluble calcium. From this fact they concluded that calcium plays an important rôle in maintaining the normal blood sugar level. In a previous (2) paper on guanidine tetany, we showed that the blood sugar could not be restored to normal by the injection of calcium lactate.

This hypoglycemia may be different from that in parathyroidectomy in which the increase of guanidine is more gradual than in guanidine tetany. Moreover, rabbits used in our experiments are less resistant to acidosis than the dog (44), and also the assimilation limit for injected glucose is lower in the rabbit (45) than the dog. These facts may explain the failure to restore the normal blood sugar level.

In looking over the literature on the influence of calcium on carbohydrate metabolism, we see the production of diabetes with calcium-poor diet by von Bunge (46), and the work of Underhill and Closson (47) on checking the glycosuria caused by continued administration of sodium chloride by the intravenous injection of calcium chloride. It is also reported that calcium administration inhibits the glycosuria caused by epinephrine (48), and the administration of sodium salts such as the oxalate or phosphate which precipitates calcium provokes the hypoglycemia (49, 50).

In considering the above facts it appears true that calcium plays an important rôle in regulating blood sugar content, but whether this is direct or indirect action is not known. In the second group of rabbits in Table III the blood sugar content shows no change even in cases where the calcium decreases to half of its normal content, as in Rabbits 23 and 26. In Rabbit 21, which

had a long continued acidosis, calcium diminished markedly, but the blood sugar was practically unchanged. On the other hand, after guanidine injection in this animal the first specimen showed a high hyperglycemia caused by the dyspnea.

From this evidence we may consider that calcium indirectly influences the sugar-regulating mechanism. Here we recall that in parathyroidectomized animals the assimilation limit for dextrose introduced by mouth or subcutaneously (51, 52, 53, 54) is lowered. This may indicate that the oxidation process is lessened in this condition. It might be correlated with the condition of acidosis since this is supported by the work of Chvostek (55) and Munk (56) in which they found that the oxidative power of the body is lessened in acid intoxication. This may induce the hypoglycemia in tetany.

#### SUMMARY.

1. The content of phosphate in the serum of normal rabbits runs from 2 to 4 mg. per 100 cc. of serum. In guanidine tetany caused by a large dose of the drug it increases in some cases to five times the normal and in almost all cases it is somewhat increased. In most cases in which several small doses were administered the phosphate increases though it remains normal in a few cases.

2. The normal calcium content of rabbit blood varies between 11 and 13 mg. per 100 cc. of serum. In guanidine tetany where the phosphate increased in the blood, the calcium decreased somewhat later. In mild, long continued, acidosis, the calcium content decreased to about half the normal value.

3. The ratio calcium : phosphate in the blood is decreased in almost all cases of guanidine tetany.

4. In the first group of rabbits, in which was injected a large dose of guanidine, hypoglycemia, with or without diminution of calcium in the same sample of blood, was manifested, whereas the phosphate content rises distinctly above the normal in all cases. In the second group of rabbits, injected with small doses at intervals, the sugar content of all specimens remained unchanged even in cases in which the calcium diminished distinctly to a value half of the normal. A high phosphate content accom-

panied the low calcium. From these facts we may deduce the proposition that calcium is not concerned in the direct control of carbohydrate metabolism in guanidine poisoning but that some other intermediary metabolic change plays a part in this phenomenon. The hypoglycemia induced by guanidine is accompanied by acidosis. Long continued acidosis so alters the metabolism that it is difficult to induce hypoglycemia.

#### CONCLUSION.

The administration of guanidine to the animal body induces a condition of severe acidosis with the retention of phosphates, a decrease of calcium in the blood, and a hypoglycemia. These phenomena are also observed in tetania parathyreopriva. Further, the symptoms and all other physiological evidences in guanidine tetany are almost the same as in tetania parathyreopriva. Since the discovery of the large increase of guanidine bases in tetania parathyreopriva and idiopathic tetany, it is possible that the fundamental cause of tetany is the increased formation of guanidine nitrogen brought about by the disturbance of the function of the parathyroid.

The author acknowledges his indebtedness to Professor F. P. Underhill for his advice and interest throughout the course of this work.

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## VITAMINE STUDIES. II.

### DOES WATER-SOLUBLE VITAMINE FUNCTION AS A CATALASE ACTIVATOR?\*

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(Received for publication, October 11, 1918.)

#### INTRODUCTION.

In a recent paper (1) we have attempted to show that avian polyneuritis is accompanied by a lowering of the catalase activity of the liver, kidney, pancreas, heart, breast muscle, lung, and blood. We also pointed out that the catalase content of tissues, in polyneuritic pigeons which had received water-soluble vitamine prepared from wheat embryo, showed a tendency to return to normal shortly after the administration of the food hormone which McCollum and Kennedy (2) have designated as "water-soluble B." It was also observed (1) that pigeons which are relatively immune to polyneuritis possess tissues, even under conditions of inanition, which are practically normal in their catalase content.

We have therefore stated that an apparent relationship exists between oxidative processes and vitamine storage in avian polyneuritis. Birds which apparently had no vitamine became polyneuritic and possessed tissues low in this oxidizing enzyme. Inasmuch as the feeding of water-soluble B brought about normal catalase content of tissues, accompanied by improvement in the condition of the bird, it would seem reasonable to suppose that the vitamine had more or less to do with the oxidative processes. Where pigeons have lived for a considerable time on diets of polished rice and have been found to possess no symptoms of

\* Published with the approval of the Director as Paper 135, Journal Series of the Minnesota Agricultural Experiment Station.



polyneuritis, and yet have been found to possess tissues which are normal in catalase, although the birds have lost in body weight, we have ventured the conclusion that these birds have been able to store larger quantities of water-soluble B in their tissues, previous to the rice diet, than the more susceptible pigeons were able to do.

Inasmuch as the water-soluble vitamine evidently causes increase in catalase production, we have conducted a few preliminary determinations to ascertain whether the stimulating action is direct or indirect.

Burge, Kennedy, and Neill (3) have shown that thyroid feeding affects the catalase activity of tissues, and Burge (4) has shown that this is also true for many food materials. Alcohol (5), when introduced into the stomach, caused an increase in blood catalase, but when alcohol was introduced directly into the blood, a decrease in blood catalase occurred. Kennedy and Burge believe (6) that the major portion of blood catalase arises in the liver, where it is stimulated to activity, or greater production at least, by pancreatic hormones, for pancreatectomy accomplished a lowering of blood catalase, while electrolytic stimulation of the splanchnic nerves caused a pronounced increase. Loevenhart (7) noted an increase in the output of oxygen when pancreas and liver and muscle and liver were combined in hydrogen peroxide. More recently Burnett (8) has made quantitative measurements on various tissues and has found that blood and liver accelerated the catalase activity of muscle tissue, and he therefore advanced the tentative explanation that the acceleration was due to an internal secretion which acts as a catalase activator. In order to ascertain whether our vitamine extracts possessed this property of direct stimulation, the following experiments were conducted.

#### EXPERIMENTAL.

A polyneuritic pigeon was killed in the early stage of the disease, and the liver, which weighed 4.806 gm., was washed free of blood with 0.9 per cent salt solution. The liver was minced and suspended in distilled water for 3 hours. The water suspension was filtered and washed, the filtrate being made to 100 cc. volume. Portions of 5 cc. each of the liver extract were placed in the cat-

alase apparatus described in a previous paper (1), and the oxygen measurements were made at the end of 10 minute periods. The bottle in every case contained 100 cc. of neutral hydrogen peroxide (3 per cent) and was agitated at the rate of 110 double shakes per minute. All measurements were made at 20°C. and 740 mm. pressure. Averages of several determinations on the 5 cc. samples of liver extract gave the following results:

Sample No.	cc.
1.....	300
2.....	305
3.....	307
4.....	302
Average.....	303

Three different vitamine extracts were used in this work, all of which had shown curative properties in our work on pigeons. Vitamine Extract I was prepared from wheat embryo and evaporated in the presence of dextrin, yielding cream-colored powder. Vitamine Extract II was also prepared from wheat embryo but was in the form of a concentrated syrup. Vitamine Extract III was a powdered sample prepared from corn pollen, with an admixture of dextrin.

None of the vitamine extracts showed any activity toward hydrogen peroxide. Determinations were made on the liver extract, using 5 cc. samples, except that the various vitamine extracts were introduced into the hydrogen peroxide bottle. All determinations gave negative results. Duplicate determinations on 5 cc. samples of the liver extract in the presence of vitamine extracts gave the following figures:

5 cc. liver extract + Vitamine Extract I	= 300 cc. O <sub>2</sub>
5 " " " + " " II	= 305 " "
5 " " " + " " III	= 296 " "
Average of liver extract alone	= 303 " "

#### CONCLUSION.

The results indicate that water-soluble B does not act as a direct activator of catalase, but instead probably (on account of its physiological properties) stimulates the organism to greater production of catalase.

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### VITAMINE STUDIES. III.

#### OBSERVATIONS ON THE CURATIVE PROPERTIES OF HONEY, NECTAR, AND CORN POLLEN IN AVIAN POLYNEURITIS.\*

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#### INTRODUCTION.

Since the United States Food Administration first showed the necessity of conserving the available supply of cane and beet sugar, much has been written to show that one way to conserve sugar would be to encourage consumption of larger quantities of syrups and honey. Much of the printed matter is designed for the general reader and, of course, many extravagant statements have been made, some of which have not accomplished the purpose for which they were written, but in the main, much real good has been attained.

In extolling the virtues of honey some writers in the past have pointed out that, although honey is not as economical as commercial sucrose, it is superior to ordinary sugar on account of its laxative action, fuel value, and medicinal properties. More recently honey producers have been asking whether honey contains vitamins. Inquiry shows that statements in the affirmative have been made in popular articles, but we have been unable, up to the present time, to find any record of investigational work in this field.

On account of the importance of this question at the present time, we have conducted experiments, which will be described

\* Published with the approval of the Director as Paper 136, Journal Series of the Minnesota Agricultural Experiment Station.

later, attempting to prove or disprove the presence of vitamins in honey.

A study of the habits of the honey-bee shows that there are, generally speaking, two types of food used by the bee, floral nectar and pollen. The floral nectar is brought to the hive in the honey stomach of the bee and deposited in the cell where it is evaporated later to honey. Most of the pollen is carried from the flower to the hive in the pollen baskets or corbicula situated on the tibiae of the third pair of legs (1); it is also transported to a much lesser degree by clinging as a dust to the body hairs. This pollen is moistened and packed into certain cells and is known as bee bread. Unpublished data in this laboratory indicate that the pollen is probably moistened with nectar or diluted honey. It is from this bee bread and honey that the larval food of the future generations is prepared.

In planning the experimental work it was felt that, should honey contain vitamins, there would be a greater possibility of finding the water-soluble vitamin in honey than its fat-soluble prototype, due to the very nature of honey which is high in water-soluble substances but low in fatty materials.

It was first pointed out by McCollum and Kennedy (2) and later corroborated by Drummond (3) that the substance found in seeds, seed embryos, thin leaves of plants, and potato juice, which cured avian polyneuritis and assisted in the general well being of growing animals, is probably one and the same substance. This has been called "water-soluble B." It was with the view of studying honey with reference to this vitamin that the following experiments were conducted.

#### EXPERIMENTAL.

The honey used in this experiment was a clear sample of strained honey obtained while basswood and white clover were in full bloom. The nectar was obtained largely from white clover, although basswood was in bloom at the time but was not being visited by many bees. This material was obtained by centrifuging the uncapped combs, newly filled with nectar, before time had elapsed for evaporation to take place. Our reason for using nectar was to study the effect of evaporation and "ripening" of honey upon its vitamin content, should vitamins be found to be present.

Microscopical examination of the nectar and honey showed a few grains of pollen to be scattered through the mass. Consequently it was thought that the water-soluble vitamine content of pollen should be taken into consideration also. Fortunately there were available for this work several pounds of corn pollen which had been collected for other experimental projects in this laboratory. It was assumed that corn pollen would be representative of other pollens in this regard and therefore it was used because of the quantity easily available.

On June 29, 1918, fifteen pigeons were placed on a diet of polished rice and these were fed, watered, and weighed in the same manner as those in preceding experiments (4). Pigeon 26 became polyneuritic on the 20th day of rice feeding and was fed about 20 gm. of basswood-clover honey (diluted) at about 9.30 a.m. At noon on the same day the bird was found dead. Similar results were obtained when Pigeon 27 became polyneuritic on the 22nd day, except that the bird did not die so quickly as Pigeon 26.

Funk and von Schönborn (5) have advanced the hypothesis that vitamines have a distinct effect on carbohydrate metabolism, and Vedder and Clark (6) are of the belief that fowls with high metabolic activity require larger amounts of vitamines and succumb more promptly to diets of polished rice.

Theiler and his coworkers (7) and Braddon and Cooper (8) found that forced feeding brought on the disease much more quickly than when the pigeon was allowed to starve itself partially. Our work is in agreement with this.

It would appear, therefore, that Pigeons 26 and 27 might have died because of overloading of the oxidative mechanism during a low vitamine feeding. Seidell (9) and Emmett and McKim (10) have shown that vitamines are adsorbed upon colloidal suspensions of siliceous earths, and this method has been used by them to separate the vitamines from other materials. With this in mind, 2 pounds of basswood-clover honey were dissolved in water and shaken for about an hour with Lloyd's reagent,<sup>1</sup> after which the siliceous earth was filtered and washed until it was no longer

<sup>1</sup> This highly adsorptive siliceous earth was generously supplied by Professor Uri Lloyd of Cincinnati.

sweet to the taste. This paste was transferred to a graduated flask and made to 200 cc. volume with water. 10 cc. portions, representing 45 gm. of honey, were fed at each feeding when needed.

Pigeon 23 showed symptoms of polyneuritis on the 19th day and was fed 10 gm. of honey in the morning. By 3 o'clock in the afternoon the bird was acutely polyneuritic with the head drawn over its back. At 5 o'clock the same afternoon a 10 cc. portion of the Lloyd's preparation from honey was administered. The next morning the pigeon was walking weakly about the cage. Toward evening the bird was much stronger, at which time another 10 cc. portion of the preparation was fed. Thereafter ground rice was fed daily with a 10 cc. portion of the Lloyd preparation, and no particular change could be noted. The bird remained on its feet but walked with difficulty. After 10 days a small amount of vitamine extract of wheat embryo was fed to this pigeon and recovery was immediate, showing that the vitamine content of the honey was relatively low compared to that in the embryo of wheat.

Similar results were obtained on Pigeon 20, with the Lloyd preparation from honey, except that the curative properties of the preparation were even less evident than in the case of Pigeon 23.

The vitamine from 2 pounds of nectar was adsorbed on Lloyd's reagent, and made to 200 cc. volume. In this case also, 10 cc. represented 45 gm. of nectar. This preparation was fed to Pigeons 13, 16, 17, 18, 21, and 22 as soon as they became polyneuritic. The pigeons were forcibly fed as soon as they refused the polished rice.

We were unable to see improvement in any of the birds receiving the nectar preparation; all birds so treated died almost immediately or were cured by changing from this diet to one high in water-soluble vitamine.

The vitamine extract from the pollen was a dry powder prepared by extracting pollen in hot 50 to 65 per cent alcohol and evaporating this on dextrin at a relatively low temperature over a steam-heated sand bath in the air current from an electric fan.

Without going into detail regarding the efficiency of the vitamine extract prepared from corn pollen, it is sufficient to say that all polyneuritic birds which received this preparation recovered very quickly. In the case of Pigeon 15 we were able to bring on symptoms of polyneuritis three successive times and then to

cause the symptoms to disappear each time by a pinch of the pollen extract. Pollen extract was also fed to Pigeons 16 and 18 which were dying, due to the lack of vitamine in the Lloyd preparation from nectar. In both of these cases the pigeons improved sufficiently to fly to their perches within 36 hours.

#### CONCLUSIONS.

Honey contains a small amount of water-soluble vitamine, but the amount is negligible. It is probable that the slight anti-neuritic properties of honey are overbalanced by the large amount of carbohydrate present, for the presence of vitamins was made evident only upon removing the sugars by adsorbing the vitamins upon siliceous earth. There was very little evidence of the presence of water-soluble vitamine in the dilute unevaporated nectar. Corn pollen is relatively rich in water-soluble vitamine and it is possible that the small amount of this in honey may have its origin in the pollen of flowering plants, either in the form of pollen grains adventitiously present, or in the digested products, which might be introduced by the salivary juices of the bee.

I am indebted to Mr. Ferdinand A. Collatz for assistance in feeding and weighing the pigeons.

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## A METHOD OF MEASURING THE ELECTRICAL CONDUCTIVITY OF LIVING TISSUES.

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(Received for publication, October 28, 1918.)

The purpose of this paper is to describe the method employed by the writer to determine the conductivity of living tissues.

This method originated as the result of studies on protoplasmic permeability. Early in these investigations the writer realized that quantitative experiments were essential to progress and undertook to develop accurate methods of measurement. The first experiments in this direction were made by plasmolysis. In connection with this the question arose whether salts penetrate as ions or as undissociated molecules. It is evident that the greater the permeability of the protoplasm, the more easily will an electric current pass through it. Hence if we can measure the electrical conductivity of the protoplasm we can measure its permeability to ions. For this purpose determinations of the conductivity of plant tissues were undertaken. The method which was devised for this purpose was developed without knowledge of methods which had previously been used for animals and differed from them. It proved to be accurate and convenient and was extensively used in subsequent investigations.

The method consists in placing the tissues between two electrodes and measuring their electrical resistance by means of a Wheatstone bridge. It is evident that some of the current must pass between the cells, flowing in the intercellular substance or spaces, but it has been shown by experiments on *Laminaria*<sup>1</sup> that a part of the current flows through the protoplasm.

It is desirable that the intercellular space or substance shall be constant in amount. This is the case in tissues, such as those of *Laminaria*, where the cell walls are of a firm consistency and

<sup>1</sup> Cf. Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 485.

do not change during the experiment.<sup>2</sup> On the other hand many flowering plants present difficulties, since the spaces between the cells are largely filled with gas, which is displaced to a varying extent when the tissue is placed in a solution, with the result that the conductivity is altered. In such cases we must select material in which the displacement is very slow or else we must get rid of the gas at the start by submerging the tissue and evacuating by an air pump.

As the writer's investigations were concerned with alterations in permeability it was necessary to provide for quick changes of reagents and for rapid penetration. This was accomplished by the use of thin sheets of tissue. For example it was found that when *Laminaria* was transferred from sea water to sea water diluted with an equal volume of distilled water, diffusion was practically completed in 5 minutes; this was also the case with the other material used in his investigations.

It is desirable that the thin sheets of tissue should be stiff enough to be handled easily and that they should not adhere to each other but should tend to separate spontaneously (this is assisted by a slight curvature of the pieces).

The cell walls should be thin and free from spaces containing gas. The material should be able to stand laboratory conditions and the manipulation required by the experiments. It is desirable that it should be available throughout the year. All these requirements are so admirably fulfilled by the marine alga *Laminaria agardhii* (a common kelp of the Atlantic coast) that it was largely used. It forms fronds several feet in length, 3 to 6 inches wide, having somewhat the consistency and thickness of a thin leather belt. It remains in normal condition in the laboratory for several weeks if kept in sea water (near 0°C.) and is not injured by the pressure and the weak electric currents to which it is subjected during the experiments.

Most of the experiments were made by means of the apparatus shown in Fig. 1. This will be called Type A in order to distinguish it from other types to be described later. It consists of two platinum electrodes,<sup>3</sup> A, sealed into glass tubes, B, which are

<sup>2</sup> Plasmolysis must be avoided since this increases the spaces between the protoplasmic masses.

<sup>3</sup> These are covered with platinum black.

filled with mercury and into which dip copper wires, C, which go to the Wheatstone bridge. These tubes are contained in electrode holders of hard rubber, D, through which pass a rod, E, and a long screw, F, by means of which the electrode holders may be drawn toward each other and held firmly in any desired position. This screw fits into an internal screw contained in the electrode holder at the right. This is not the case with the electrode holder at the left and in consequence this electrode holder is drawn toward the other only when the block, M, is fastened in place by the set screw, N, and the screw, F, is turned in the proper direction.

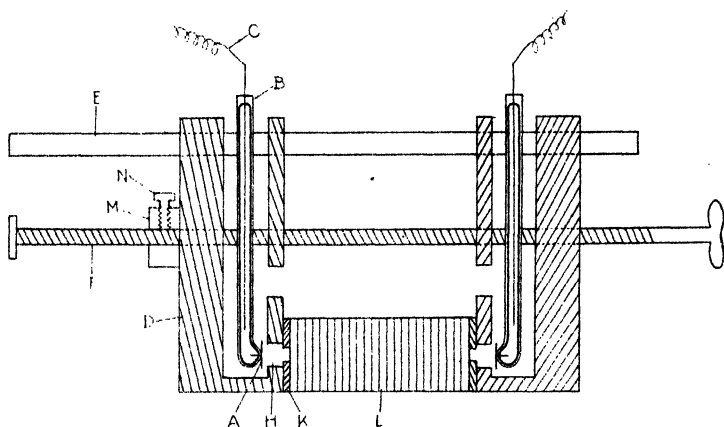


FIG. 1. Apparatus for determining the electrical conductivity of living tissue. The disks of tissue, L, are packed together like a roll of coins. At each end is a platinum electrode, A, fastened in an electrode carrier, D. By means of the screw, F, the electrode carriers can be drawn together, compressing the tissue and holding it firmly in place.

An end view of an electrode holder, D, is shown in Fig. 2. Its lower portion (which contains the platinum electrode) is shown inserted into a hard rubber support, G. The support is pierced by a series of seven holes (arranged in a circle as shown in the figure) each of which receives the end of a glass rod about 9 inches long, the other end of each rod being fastened in a similar support. The circle (dotted line) just inside the seven small circles represents a disk of tissue inserted between the glass rods with its

surface at right angles to them. The smaller circle, H, in the center represents an opening in the electrode holder through which the current passes from the platinum electrode to the disks of *Laminaria*. The arrangement is shown in Fig. 1, where H represents the opening and L represents the disks. Before reaching the disks the current passes through, K, (Fig. 1), a hard rubber disk (with an opening in the center) which provides mechanical support for the tissue.

The disks are cut from the fronds by means of a cork borer and have about the diameter and thickness of a silver quarter. They are packed together like a roll of coins (about 80 in all) and form a mass of just the right elasticity for the purpose of the experi-

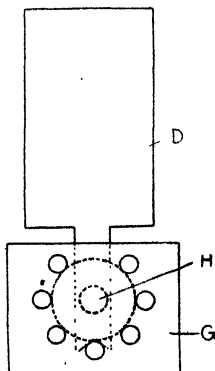


FIG. 2. Electrode carrier, D, seen from the end, resting upon a frame, G, in which are set glass rods (seen in section, as a series of circles) which hold in place the disks of tissue (seen in section as a dotted line).

ment. They are held firmly in place by the glass rods which surround them and by the electrode holders which press against them at either end. At the same time the spaces between the glass rods allow free circulation of liquid.

Each disk is placed in sea water as soon as it is cut;<sup>4</sup> from this the disks are transferred to the support G, which is submerged

<sup>4</sup> It was at first thought that cutting might injure the tissues at the edge of the disk sufficiently to interfere with the results, but experiments proved that this is not the case. Not only do the cells adjoining the cut surface live as long as those in the center of the disk but it is found that experiments (made by another method) on intact fronds give the same results as experiments on the cut disks.

in sea water. They are arranged inside the glass rods (being packed together, like a roll of coins, to form a cylinder) by means of forceps, and care is taken to see that no bubbles of air are caught between them or in the opening in K. The electrode holder is now placed in position and inspected to see that no air is caught in the space around the electrode or in the opening at H.

The electrode holders are now pressed against the ends of the roll of disks, the block, M, is firmly fastened by means of the set screw, N, and the screw, F, is turned until the electrode holders are tightly clamped against the roll of disks. The pressure used in this operation should be fairly uniform.<sup>5</sup>

The apparatus is now lifted out of the sea water<sup>6</sup> and allowed to drain<sup>7</sup> for a definite time (not over 1 minute) after which the resistance becomes practically constant.

The current passes for a short distance through sea water before reaching the disks. There is a film of sea water between each pair of disks and likewise a film around the cut edges. Otherwise the current passes only through the tissue.

As soon as the resistance has been measured the apparatus is replaced in sea water; the set screw, N, is loosened so that the electrode holders can be moved apart and the disks separated from each other by means of forceps. After standing for a few minutes in sea water the resistance is again determined. The disks are then separated as before and allowed to stand in sea water. This procedure is continued until it becomes evident that the resistance is practically stationary.<sup>8</sup>

The apparatus is then transferred to another solution (*e.g.*,

<sup>5</sup> It was at first thought necessary to use a dynamometer but it was found that the operator soon becomes so proficient as to make it unnecessary. The resistance is very little affected by variations in pressure.

<sup>6</sup> In the earlier experiments the resistance was taken with the cylinder submerged in sea water, and this may be preferable in special cases.

<sup>7</sup> Each support rests on a block of paraffin. Care must be taken that there is no conduction between the blocks; *e.g.*, along the wet surface of the table.

<sup>8</sup> Unless this is the case the material is rejected. With good material the resistance remains stationary for a long time (in one experiment it remained so for 10 days). See Osterhout, *Bot. Gaz.*, 1915, lix, 242.

NaCl 0.52 M) having the same conductivity (and temperature<sup>9</sup>) as the sea water. There should be at least 1,500 cc. of solution, contained in a shallow dish of glass or enameled ware. The disks are at once separated by means of forceps and thoroughly rinsed in the new solution, the whole apparatus being moved about in the dish to secure thorough mixing. By means of a medicine dropper the sea water around the platinum electrodes is thoroughly washed out. In some cases it is desirable to transfer to a second dish to ensure against contamination by sea water.

By this means a very rapid change is effected and, as the disks are thin, diffusion is soon completed (this is often the case in 5 minutes and should not in any event require more than 10 minutes). Since the outward diffusion of salts may take place at a different rate from the inward diffusion there may be an apparent rise or fall of resistance in consequence. This effect lasts but a short time and is found in dead as well as in living tissue. It is therefore easy to guard against error due to such causes.<sup>10</sup>

Certain additional precautions deserve mention. Only sound material should be chosen and disks should be cut from portions of the frond which are smooth and firm.<sup>11</sup> The best disks are those which curve slightly, so that when in the apparatus they spontaneously separate whenever the pressure is removed.

The resistance of the disks at the ends is much greater than that of those in the middle since the current spreads out after issuing from the small opening,<sup>12</sup> H, (Fig. 1) in the rubber disk. For this reason the best disks of tissue should be placed at the ends and their positions should not be changed. Care should be taken that they are not cut or injured by contact with the edges of the opening in the rubber disk.<sup>13</sup> The inequality between the

<sup>9</sup> All readings should be made at the same temperature or, if this is not practicable, should be corrected to the standard temperature. For the temperature coefficient see Osterhout, *Biochem. Z.*, 1914, lxvii, 272.

<sup>10</sup> Cf. Osterhout, *J. Biol. Chem.*, 1918, xxxvi, 489.

<sup>11</sup> Portions of the frond which are covered with reproductive tissue should not be used.

<sup>12</sup> It results from this that the resistance does not increase in direct proportion to the number of disks. If we plot the resistance as ordinates and the number of disks as abscissæ, we obtain a curve which is concave toward the base line. The curve is approximately logarithmic.

<sup>13</sup> These edges may be rounded by filing. A soft rubber disk may be placed between the hard rubber disk and the tissue.

disks at the end and in the center may be minimized by introducing at intervals rubber disks provided with openings in the center (Fig. 3). This is in many cases desirable and enables us to get a high resistance with less tissue.

Care must be taken to see that liquid does not leak out of the space around the electrodes while the apparatus is out of the liquid. If a leak should occur fresh liquid may be added by means of a medicine dropper. With a proper adjustment of the rubber disks and sufficient tissue to give elasticity no leakage should occur.

In regard to the accuracy of the readings it may be said at the outset that under favorable conditions successive readings on the same material do not vary more than 1 per cent from the average. This is as great accuracy as can ordinarily be hoped for in

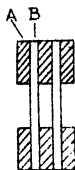


FIG. 3. Hard rubber disks, A, alternating with disks of tissue, B (all seen in section).

biological work and there is no object in striving to get greater accuracy than this in the apparatus itself.

It is desirable to get a good minimum in the telephone and the insertion of capacity in the circuit may help to accomplish this. The frequency is also of some importance in this connection. The writer has found a thousand cycles convenient; this is furnished by an apparatus obtained from Leeds and Northrup.

The use of the ordinary lighting circuit (60 cycles) with a vibration galvanometer is recommended by Green.<sup>14</sup> An alternating current galvanometer may be used in connection with a recording device if desired.<sup>15</sup>

<sup>14</sup> Green, N. B., *Am. J. Bot.*, 1917, iv, 411.

<sup>15</sup> Cf. Weibel, E. E., and Thuras, A. L., *J. Ind. and Eng. Chem.*, 1918, x, 626.



For details regarding apparatus the reader is referred to the papers of Hibbard and Chapman,<sup>16</sup> of Washburn,<sup>17</sup> and of Taylor and Acree.<sup>18</sup>

We may now turn to another form of apparatus which may for convenience be called Type B. Fig. 4 shows one end of the apparatus, which consists of an electrode holder, A, and a series of glass cells, B, C, etc. The electrode holder consists of a glass tube provided with side arms for the admission of the electrode tube, D, (which is similar to the tube used in Type A) as well as

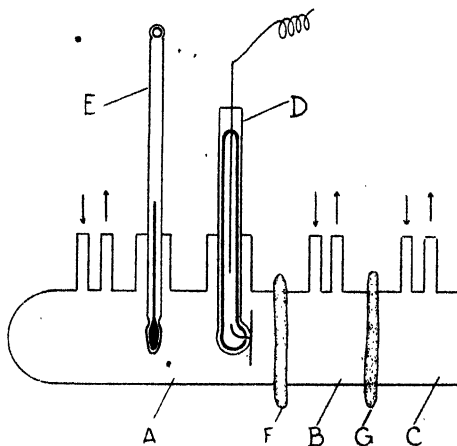


FIG. 4. Electrode carrier, A, consisting of a glass tube provided with a series of side tubes to hold an electrode tube, D, and a thermometer, E; also an inlet tube and an outlet tube. To the right two glass cells, B, C, each with an inlet tube and an outlet tube, with disks of tissue, F and G.

of a thermometer, E. In addition there is an inlet tube and an outlet tube by means of which the solution may be changed. Each of the glass cells, B, C, etc., has a similar inlet tube and

<sup>16</sup> Hibbard, R. P., and Chapman, C. W., *Michigan Agric. College, Technical Bull.* 23, 1915.

<sup>17</sup> Washburn, E. W., and Bell, J. E., *J. Am. Chem. Soc.*, 1913, xxxv, 174. Washburn, E. W., *ibid.*, 1916, xxxviii, 2431. Washburn, E. W., and Parker, K., *ibid.*, 1917, xxxix, 235.

<sup>18</sup> Taylor, W. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2415, and previous papers in the same *Journal*.

outlet tube. Each outlet tube has a rubber connection through which liquid can be discharged without wetting the cells. All of the inlet tubes are connected (by rubber tubing and a system of Y-tubes) to the same funnel so that all the cells can be filled simultaneously.

\* The edges of the glass cells are ground in a plane exactly at right angles to the long axis of the cell. When pieces of *Laminaria* are placed between them (as at F and G) and they are pressed together, a tight joint is formed. The series of glass cells (with pieces of material) and an electrode carrier at each end are placed in a V-shaped trough with rigid ends; at one end is a screw by means of which they can be forced together and held with any desired degree of pressure. At the places where the pieces of material are located, the trough is cut away so that they do not come in contact with it. Care is taken to keep the current from leaking along the trough (its surface is covered with paraffin).

The current therefore flows through the glass cells and through the pieces of material placed between them.

The advantages of this type of apparatus are: (1) the end pieces do not have more resistance than those in the middle; (2) the solutions may be changed without disturbing the material.

Types A and B may be combined by substituting disks of *Laminaria* for the glass cells.

Type C is shown in Fig. 5. It consists of two electrode carriers similar to those in Type A. The material is shown at M, its edges being completely surrounded by vaseline, V, V, so that the current cannot leak out. In many cases it is preferable to use chicle, grafting wax, or art gum in place of vaseline. The apparatus remains partly submerged (the water line being indicated at W, W), thus keeping the temperature more nearly constant. The solutions are changed by siphoning through the openings which admit the electrode tubes. This makes it unnecessary to unscrew and separate the electrode carriers during the experiment.

Type D is shown in Fig. 6. It permits the use of intact plants. One end of the plant is inserted into each of the cells A and B and held in place by a split rubber stopper. The cells A and B are filled with solution. The free portion of the plant is bathed in any desired solution until a reading is to be taken, when the

solution is allowed to drain off and the reading is made. Care should be taken to prevent the current from leaking through or around the stopper.

The part of the frond which is contained in the stopper and in the cell may be killed to lessen its resistance.

Material which is too soft to be handled in the manner recommended for *Laminaria* may be treated as follows: If it forms sheets or membranes it may be fastened to thin disks of hard

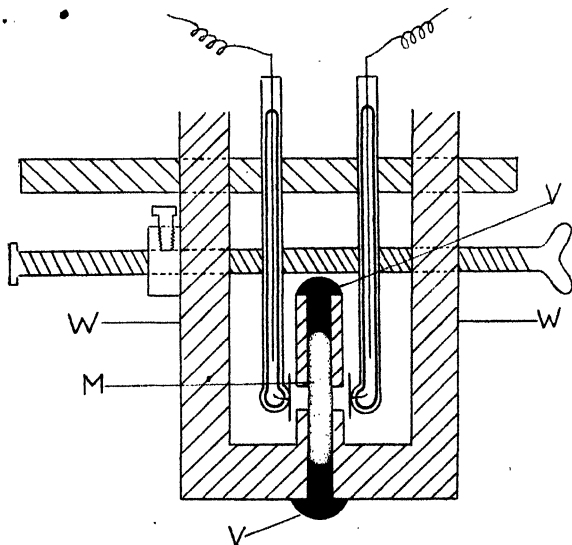


FIG. 5. Disk of tissue, M, the edges surrounded by vaseline, V, V, with an electrode carrier on each side.

rubber<sup>19</sup> provided with a central opening as shown in Fig. 7, in which A represents the rubber disk (seen in section), B the material, and C another disk of thin rubber or celluloid. These are fastened together by rubber bands, D. For this purpose three projecting knobs are provided as shown in the surface view at the left of Fig. 7. The disk is placed in the frame described under Type A, and the knobs fit in between the glass rods in the manner

<sup>19</sup> The edges of each piece of tissue are protected by vaseline.

shown in Fig. 7 (where the rods appear in section). Every other disk is turned upside down so that the knobs of adjacent disks

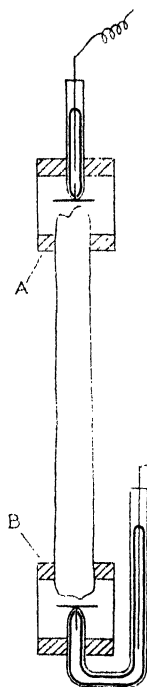


FIG. 6.

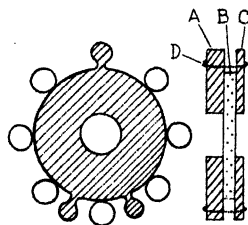


FIG. 7.

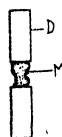


FIG. 8.

FIG. 6. Two glass cells, A and B, each provided with an electrode; a strip of tissue is stretched between them.

FIG. 7. A disk of hard rubber, A, one of tissue, B, and one of celluloid, C, tied together with rubber bands, D (all seen in section). Surface view at the left.

FIG. 8. Disk of hard rubber, D, with a mass of tissue, M, wedged in the central opening (seen in section).

do not touch and interfere with the close packing of the disks. The disks are treated precisely like the disks of *Laminaria* as described under Type A.

Material which cannot be handled in this way may be treated as shown in Fig. 8, where D represents a hard rubber disk with a central opening into which the material is tightly wedged. The disks are then handled like so many disks of *Laminaria*.

#### SUMMARY.

A method of measuring the electrical conductivity of living organisms is described which can be applied to pieces of tissue or to intact organisms. Under the most favorable circumstances measurements made by this method do not vary more than 1 per cent from the mean.

# THE EFFECT OF THE MATERNAL INGESTION OF DESICCATED PLACENTA UPON THE RATE OF GROWTH OF BREAST-FED INFANTS.

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(From the Department of Anatomy, Harvard Medical School, Boston.)

(Received for publication, October 8, 1918.)

During a series of experiments designed to show whether or not the maternal ingestion of desiccated placenta would have an effect upon the chemical composition of the milk produced during the early stages of lactation,<sup>1, 2</sup> a comparison of the growth of the infants feeding upon the milk produced during its administration with the growth of those subsisting upon milk from mothers who had not been given the material,<sup>3</sup> led to the idea that the feeding of this substance to the mothers had an effect upon the rate of growth of their breast-fed infants entirely aside from the per cent change in the determined constituents.

Since the number of observed cases was too small to give more than the indication of a tendency, I decided to make a more extensive study of the matter, in order to determine definitely whether placenta tissue *per se*, when fed to nursing mothers, contains a substance or substances affecting the rate of growth of the breast-fed infants, it being a well known fact that milk may contain as such, or slightly changed, various substances, ordinarily foreign to its make-up, ingested by the mother.

The cooperation and courtesy of the staff and nurses of the Boston Lying-In Hospital made possible the carrying out of this plan, and the following is a brief report of the results.

In order to avoid complications in recording, all patients were given the desiccated placenta prepared as described in an earlier publication,<sup>2</sup> and in the same dosage, *e.g.* 10 grains in capsules,

<sup>1</sup> Hammett, F. S., *J. Biol. Chem.*, 1917, xxix, 381.

<sup>2</sup> Hammett, F. S., and McNeile, L. G., *J. Biol. Chem.*, 1917, xxx, 145.

<sup>3</sup> Hammett and McNeile, *Science*, 1917, xlv, 345.

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*t.i.d.* From these, only those mothers were chosen whose parturition course was normal, and only the weights of those infants are utilized whose sole source of nourishment was the maternal breast. The diet of the mothers and the method of weighing of the infants were the same as reported in the paper on the normal growth capacity of infants at this Hospital,<sup>4</sup> thus standardizing the experimental conditions for a study of this type.

<sup>1</sup> In view of the results obtained in a study of the relationship between weight at birth and growth capacity,<sup>4</sup> and for purposes of comparison, the subjects of this experiment were also divided

TABLE I.

*Comparison with the Normal of the Per Cent Change in Weight from the 1st Day of Infants Subsisting upon Milk Produced under the Influence of Maternally Ingested Desiccated Placenta.*

Group.	Weight.  <i>lbs.</i>	Day.											
		3		5		7		9		11		13	
		Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.
A	5-6	-3.1	-4.0	-1.2	-3.3	0.6	-0.4	4.7	1.4	8.8	3.9	12.5	6.0
B	6-7	-4.1	-4.7	-2.1	-3.2	0.4	-1.1	3.0	0.9	5.2	2.8	7.9	4.4
C	7-8	-3.7	-4.4	-2.7	-2.7	0.1	-1.1	2.3	1.0	4.3	2.7	5.9	4.6
D	8-9	-4.5	-5.1	-3.0	-3.8	-1.3	-2.4	0.3	-0.6	2.0	0.9	3.3	2.1
E	9-10	-4.5	-7.2	-4.8	-7.1	-3.3	-5.9	-3.0	-4.7	-1.7	-4.0	-1.1	-2.9
F	10-11	-5.4	-7.4	-5.6	-7.3	-3.5	-6.4	-0.9	-5.6	0.9	-4.7	1.5	-3.6

into six groups according to weight at birth; and the weights recorded on the 1st, 3rd, 5th, 7th, 9th, 11th, and 13th days after birth were utilized for the data presented. The growth of 177 infants was studied during this period.

In Table I is given a comparison of the per cent change in weight of the two general sets of subjects. Fig. 1 is a graphic representation of the mean curve of each set.

The effect of the ingestion of the desiccated placenta by the mothers on the rate of growth of the breast-feeding infants is at

<sup>4</sup> Hammett, *Am. J. Physiol.*, 1918, xlv, 396.

once apparent. In each group not only is the postnatal decline in weight less in amount, but also the gain in weight after the preliminary loss is greater in every case on every day than that recorded for the normal groups, the mean increase over the normal per cent change in weight on the 13th day being over 60 per cent.

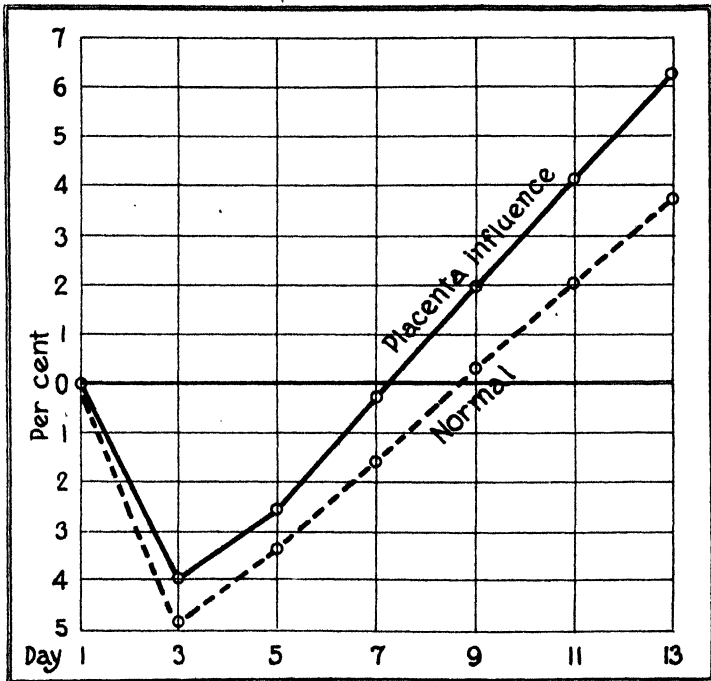


FIG. 1. Curves showing mean of per cent change in weight from 1st day of two sets of infants.

Supplementing these facts with an examination of Table II conclusively demonstrates that the rate of growth of breast-fed infants is enhanced by the maternal ingestion of desiccated placenta, for not only is the recovery to or over the initial weight generally more rapid, but the weight is almost uniformly greater.



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The growth capacity of infants subsisting upon the milk produced during the administration of desiccated placenta is also increased. This is shown in Table III. That is to say, the maternal ingestion of dried placenta tissue so stimulates the tissues of the infants feeding upon the milk produced during this time,

TABLE II.

*Comparison of the Per Cent Recovery to or over the Initial Weight of the Two Sets of Infants.*

Group.	Weight.	Day.											
		3		5		7		9		11		13	
		Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.	Placenta.	Normal.
	lbs.												
A. ....	5-6	17	19	17	29	58	50	83	62	100	75	100	82
B. ....	6-7	12	8	36	24	50	45	71	60	79	75	95	80
C. ....	7-8	0	12	27	24	54	39	78	60	87	74	89	78
D. ....	8-9	5	17	17	17	40	30	60	49	70	60	79	70
E. ....	9-10	0	2	22	5	33	15	33	20	33	30	55	35
F. ....	10-11	0	3	0	3	25	5	50	8	75	11	75	20

\* TABLE III.

*Comparison of the Growth Capacity of the Two Sets of Infants.*

Group.	Weight.	Increment from 3rd day.		Capacity.	
		Placenta.	Normal.	Placenta.	Normal.
	lbs.	per cent	per cent	per cent	per cent
A. ....	5-6	15.6	10.0	2.836	1.818
B. ....	6-7	12.0	9.1	1.846	1.400
C. ....	7-8	9.6	9.0	1.280	1.200
D. ....	8-9	7.8	7.2	0.918	0.847
E. ....	9-10	3.5	4.3	0.369	0.453
F. ....	10-11	6.9	3.6	0.657	0.343

that unit weight is able to add on greater increments of matter, from day to day, than can unit weight of infants feeding on milk from mothers not ingesting this substance.

A large series of comparative measurements of the mammæ of women taking and not taking the desiccated placenta, combined

with a study of the time of onset of full milk production, failed to show either hypertrophy of the gland or an increased milk production on the part of those women ingesting the placenta material. In view of these facts, and having in addition evidence that the increased food value of the milk produced during the administration of the dried placenta cannot compensate for the increased growth of itself,<sup>2,3</sup> and moreover since but 30 grains, or less than a gram, of the dried material was fed each day, a quantity so small as to be negligible as matter from which new tissue can be built, we conclude that there must be contained in the desiccated placenta some substance, or substances, capable of passing through the maternal organism with at least a part of its activity retained. Being secreted by the mammary glands, it is passed on to the infant in the milk, there acting as stimuli to growth. It is not illogical to suppose that these substances in the placenta *in utero* may play an important part in the growth of embryo and fetus.

#### CONCLUSION.

The maternal ingestion of desiccated placenta produces an increase in the rate of growth and growth capacity of the breast-fed infants above that normally occurring. This is due to the presence in the placenta of some, as yet unidentified, growth-promoting substance.



## ACTION OF ENZYMES ON HUMAN PLACENTA.

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(Received for publication, October 29, 1918.)

The autolysis of human placenta has been the subject of several studies. That the placenta contained an autolytic enzyme was first shown by Mathes<sup>1</sup> in 1901. Ascoli,<sup>2</sup> Bergell and Liepmann,<sup>3</sup> and Bergell and Falk<sup>4</sup> also examined placenta for proteoclastic enzymes, with positive results, though the latter were unable to state whether the enzyme belonged to the tryptic or ereptic type. On the other hand, Lochhead and Cramer<sup>5</sup> were unable to find any true tryptic or ereptic enzymes. Löb and Higuchi<sup>6</sup> also failed to prove their existence. Within recent years exhaustive studies of the Abderhalden reaction have revealed the fact, however, that the proteoclastic enzyme of the blood, whether male or female, has a pronounced action upon placenta.<sup>7</sup> The action, however, of the digestive juices upon placenta does not appear to have been examined, though the medical literature contains many references to placental feeding. As the placenta and placental products are of high importance in many theories of the etiology of the toxic disturbances of pregnancy, it appeared important to us to undertake such a study. Moreover, our own experimental work was leading us in the direction of placental feeding and it was essential for us to be certain of the digestibility of our product. We have consequently made a short study of the action of the digestive enzymes upon our placenta preparation, and then sup-

<sup>1</sup> Mathes, P., *Centr. Gynäk.*, 1901, xxv, 1885.

<sup>2</sup> Ascoli, A., *Z. physiol. Chem.*, 1902, xxxvi, 498.

<sup>3</sup> Bergell, P., and Liepmann, W., *Münch. med. Woch.*, 1905, lii, 2211.

<sup>4</sup> Bergell, P., and Falk, E., *Münch. med. Woch.*, 1908, lv, 2217.

<sup>5</sup> Lochhead, J., and Cramer, W., *Proc. Roy. Soc., Series B*, 1908, lxxx, 263.

<sup>6</sup> Löb, W., and Higuchi, S., *Biochem. Z.*, 1909, xxii, 316.

<sup>7</sup> Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377.

plemented our experiments *in vitro* with a comparative digestibility trial. We may say at once that the placenta is readily attacked by pepsin, trypsin, and erepsin. It is also readily hydrolyzed by weak acids and alkalies. We have studied its digestibility in the dog by comparing it with meat, with which it has almost the same nitrogen content. Its digestibility, under the same conditions, on a protein and carbohydrate diet, is about the same as lean meat.

## EXPERIMENTAL.

The placental substrate was prepared according to the directions of Harding and Fort.<sup>8</sup> The pepsin was a preparation of Parke Davis and Company; trypsin was prepared according to the directions of Cole<sup>9</sup> from sheep pancreas, and the erepsin

TABLE I.  
*Action of Pepsin.*

No. of flask.	Enzyme solution.	0.4 per cent HCl.	Water.	Placenta.	Time of digestion.	0.05 N NaOH per 100 cc.	Hydrolysis.	Enzymatic acceleration.	Remarks.
	cc.	cc.	cc.	gm.	hrs.	cc.	per cent	per cent	
1	2	5	33	0.3926	24	9.40	9.70	6.96	Digestion practically complete in 24 hrs. Acid exerts a slight hydrolytic effect.
2	2	5	33	0.3927	24	5.96	2.74		
3	2	5	33	—	24	4.60	—		
1	2	5	33	0.3918	24	9.60	9.88	7.18	
2	2	5	33	0.3933	24	6.08	2.71		
3	2	5	33	—	24	4.72	—		
1	2	5	33	0.3931	48	9.80	9.83	7.08	
2	2	5	33	0.3911	48	6.00	2.75		
3	2	5	33	—	48	4.64	—		
1	2	5	33	0.3941	96	10.28	11.16	8.66	
2	2	5	33	0.3925	96	5.96	2.50		
3	2	5	33	—	96	4.72	—		
1	2	5	33	0.3925	96	10.20	10.90	8.24	
2	2	5	33	0.3927	96	6.12	2.66		
3	2	5	33	—	96	4.80	—		

<sup>8</sup> Harding, V. J., and Fort, C. A., *J. Biol. Chem.*, 1918, xxxv, 29.

<sup>9</sup> Cole, S. W., *Practical physiological chemistry*, Cambridge, 3rd edition, 1913, 94.

from pig intestine by simple extraction with distilled water, according to Cohnheim.<sup>10</sup> As a method of experimental procedure, we took three flasks and placed in them the following: (1) enzyme + acid or alkali + placenta; (2) enzyme (boiled) + acid or alkali + placenta; (3) enzyme + acid or alkali. In the protocols the number of the flask will refer to the flask as just described. The flasks were sterilized before use, and toluene

TABLE II.  
*Action of Trypsin.*

No. of flask.	Enzyme solution.	0.4 per cent Na <sub>2</sub> CO <sub>3</sub> .	Water.	Placenta.	Time of digestion.	0.05 N NaOH per 100 cc.	Hydrolysis.	Enzymatic acceleration.	Remarks.
	cc.	cc.	cc.	gm.	hrs.	cc.	per cent	per cent	
1	5	10	25	0.3918	24	40.96	49.58	30.68	Digestion, as in the case of pepsin, is complete in 24 hrs. Sodium carbonate exerts a strong hydrolytic effect. All the placenta was dissolved at the end of the experiment.
2	5	10	25	0.3913	24	25.80	18.90		
3	5	10	25	—	24	16.48	—		
1	5	10	25	0.3917	24	41.48	50.33	32.20	
2	5	10	25	0.3924	24	25.60	18.12		
3	5	10	25	—	24	16.48	—		
1	5	10	25	0.3923	48	42.12	51.55	30.04	
2	5	10	25	0.3934	48	27.30	21.50		
3	5	10	25	—	48	16.64	—		
1	5	10	25	0.3954	48	42.20	51.02	29.68	
2	5	10	25	0.3940	48	27.30	21.34		
3	5	10	25	—	48	16.72	—		
1	5	10	25	0.3940	96	42.32	51.57	32.44	
2	5	10	25	0.3936	96	26.20	19.45		
3	5	10	25	—	96	16.72	—		
1	5	10	25	0.3951	96	42.62	52.02	31.45	
2	5	10	25	0.3928	96	29.60	20.56		
3	5	10	25	—	96	16.72	—		

was added as a preservative during the experiment. The flasks and contents were stoppered with absorbent cotton and incubated at a temperature of 37.5° for a definite number of hours. They were then removed from the incubator, and the enzyme was destroyed by rapidly bringing the contents of the flask to a boil and then cooling. The solution was then diluted to 100 cc., fil-

<sup>10</sup> Cohnheim, O., *Enzymes*, New York, 1912, 4.

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tered, and the extent of proteolysis in 25 cc. determined by the Sørensen<sup>11</sup> method, phenolphthalein being used as an indicator.

The results were calculated as the percentage hydrolysis of the amino nitrogen of the fully hydrolyzed placenta. The nitrogen content of the placenta was determined by the Kjeldahl method and found to be 12.46 per cent. According to Harding and Fort,<sup>8</sup> the amino-acid nitrogen (free and combined) of placenta

TABLE III.  
*Action of Erepsin.*

No. of flask.	Enzyme solution.	0.4 per cent NaCO <sub>3</sub> .	Water.	Placenta.	Time of digestion.	0.05 N NaOH per 100 cc.	Hydrolysis.	Enzymatic acceleration.	Remarks
	cc.	cc.	cc.	gm.	hrs.	cc.	per cent	per cent	
1	5	0.5	44.5	0.3657	24	7.20	13.890	13.890	Digestion practically complete in 24 hrs. With the smaller amount of sodium carbonate there is no hydrolytic effect due to that cause.
2	5	0.5	44.5	0.3905	24	4.05	—	—	
3	5	0.5	44.5	—	24	4.00	—	—	
1	5	0.5	44.5	0.3791	24	7.50	14.358	14.358	
2	5	0.5	44.5	0.3897	24	4.05	—	—	
3	5	0.5	44.5	—	24	4.05	—	—	
1	5	0.5	44.5	0.3876	48	7.90	14.334	14.334	
2	5	0.5	44.5	0.3879	48	3.95	—	—	
3	5	0.5	44.5	—	48	4.40	—	—	
1	5	0.5	44.5	0.3906	48	8.05	14.473	14.473	
2	5	0.5	44.5	0.3871	48	4.05	—	—	
3	5	0.5	44.5	—	48	4.50	—	—	
1	5	0.5	44.5	0.3994	96	8.10	14.504	14.504	
2	5	0.5	44.5	0.3944	96	4.05	—	—	
3	5	0.5	44.5	—	96	4.45	—	—	
1	5	0.5	44.5	0.3924	96	8.00	14.562	14.562	
2	5	0.5	44.5	0.3868	96	4.10	—	—	
3	5	0.5	44.5	—	96	4.40	—	—	

is 70.8 per cent of the total nitrogen present. Therefore the percentage of amino-acid nitrogen in the placenta preparation was 8.82. The percentage hydrolyses were calculated on this figure.

In the comparative digestibility trial *in vivo* a diet of potato and lean beef was supplied to a puppy. The meat was freed as far as possible from fat and connective tissue, minced, and ex-

<sup>11</sup> Sørensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

tracted with water. It was then coagulated, air-dried, and ground in a mill to a fine powder, thus resembling in its preparation and texture the placental powder. 15 gm. of meat were given per day, and the feeding was continued for 5 days. The nitrogen excreted in the urine was determined each day. A second period of 5 days then followed, with 15 gm. of placental powder

TABLE IV.

Puppy.	Nitrogen excretion per 24 hours.	
	Meat.	Placenta.
	<i>gm.</i>	<i>gm.</i>
W.	1.032	1.615
	1.689	1.503
	1.176	1.908
	1.153	1.764
	1.274	1.433
Average...	1.265	1.665
G.	0.881	1.241
	1.236	1.248
	1.172	1.319
	1.180	1.122
	1.293	1.193
Average...	1.152	1.225
N.	1.526	1.637
	1.512	1.758
	1.606	1.541
	1.602	1.474
		1.496
Average...	1.561	1.581

replacing the beef. The nitrogen content of the two is identical; both had been extracted with water and were of the same physical texture. The nitrogen excretion per day was again determined in the second period, and was found to be always slightly higher than the nitrogen of the first period. Hence it was concluded that the digestibility of placental powder is about the same as lean meat, under the same conditions. No ill effects were no-



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ticed on the animals, who devoured the rations greedily, and who also showed increase in weight during the experimental period.

15 gm. of meat or placental powder, 250 gm. of potato, 35 gm. of cornstarch, or dextrin, 5 gm. of cane sugar, were supplied each day to a puppy of about 3 to 4 months. Nitrogen content of either diet was 2.0 gm.

### SUMMARY.

Normal human placenta is readily hydrolyzed by pepsin, trypsin, and erepsin, and is readily and easily digested by the dog.

# THE MEASUREMENT OF THE ACIDITY OF BREAD.

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(Received for publication, October 23, 1918.)

The degree of acidity is of importance in bread making in that it determines the physical state of the gluten, influences the growth and the activity of the yeast, and controls the growth of many other microorganisms, such as the rope-producing bacillus *Bacillus mesentericus*. The growth of yeast and other microorganisms in turn influences the acidity of dough and of bread. A colorimetric method for measuring the relative acidity of bread is therefore suggested.

The effect of the hydrogen ion concentration<sup>1</sup> upon the hydration, the viscosity, and the degree of dissolution of gluten has been the subject of researches by Wood, Wood and Hardy, Olson, Stockholm, Upson and Calvin, Gortner and Doherty, and numerous other investigators. These authors have made observations which experiments soon to be published from this laboratory have confirmed and at certain points extended. A comparison of the results obtained from the study of gluten with such baking experiments as those of Jessen-Hansen and others seems to indicate that there is an optimum hydrogen ion con-

<sup>1</sup> The degree of acidity is best measured by the electrometric or colorimetric determination of the concentration in hydrogen ions, commonly expressed as pH.

centration for the baking of bread. As this paper goes to press the investigation of Landenberger and Morse can be cited in further support of this view.

The hydrogen ion concentration of bread is related to the original acidity and to the acid-combining power of the cereals used, to the amount of yeast, and in that the activity of yeast varies with the acidity of the medium and modifies it through the acid products of its own metabolism, to the conditions of fermentation.

Further, sufficient acidity effectively checks the growth of many other microorganisms. In general this is an important matter in the technology of fermentation; specifically in bread making it is involved in the problem of the prevention of rope. At the present time the last factor is of serious economic importance for we have found<sup>2</sup> that a large variation in the acidity of bread results from the changing formulas and different substances which the bakers are at present obliged to use. This is illustrated by the data collected in Table II of the variation in hydrogen ion concentration of the loaves of the largest Boston bakeries. Usually the wheat substitutes tend to reduce the acidity of the loaf. We have, however, also found that beyond a hydrogen ion concentration of  $10^{-5}N$  (pH 5) *Bacillus mesentericus*, which seems to be the common cause of rope, cannot develop in bread, and that its development is inhibited as the hydrogen ion concentration approaches  $10^{-6}N$ .

It has therefore seemed desirable to determine the hydrogen ion concentration of bread in a considerable number of cases, and to find a method of making this measurement which shall be at once trustworthy and sufficiently simple.

At the outset this problem is complicated by uncertainty of definition, for it is not clear what meaning is to be attached to the phrase, *the hydrogen ion concentration of bread*, and in any case this concentration cannot be directly measured so as to give absolute rather than relative values of known accuracy. Furthermore there is a considerable difference in the hydrogen ion concentration measurements, whether with concentration cell or with indicators, of aqueous extracts of bread on the one hand, and of suspensions on the other. Nevertheless experience has

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<sup>2</sup> See Bibliography, Cohn, Wolbach, and Henderson, and Henderson.

proved that consistent results, which are quite satisfactory for practical purposes and which may be provisionally expressed in terms of hydrogen ion concentration, are not difficult to obtain. For the present the more exact definition of the question may accordingly be postponed.

When a drop of the ordinary indicator solution of methyl red falls upon a slice of bread it assumes a color which may vary, according to the acidity of the loaf, from orange to red. If the loaf is ropy, the color will probably be yellow, as a result of the alkali produced in the metabolism of *Bacillus mesentericus*. Baking experiments upon dough of known but graduated acidities show that the range from orange to red corresponds to initial values of pH ranging from approximately 6.0 to 4.5.

TABLE I.

*Effect of Lactic Acid upon Hydrogen Ion Concentration of Dough and of Bread.*

Experiment 744.	0.5 N lactic acid added to 300 gm. of flour.	pH		
		Immediately upon mixing dough.	After 4 hrs. fermentation just before baking.	Bread after baking.
	cc.			
1	0		5.30	5.38
2	1.0		5.24	
3	1.6	5.55		
4	2.4			
5	3.2		4.98	
6	4.0	5.20	4.94	4.98

An experiment in which the acidity of the dough and of the resulting bread was progressively increased by the addition of lactic acid is reported in Table I. The resulting loaves clearly showed the gradient in color of methyl red.

This result has led us to make parallel observations on the hydrogen ion concentrations of aqueous suspensions<sup>3</sup> of nu-

<sup>3</sup> The suspensions of bread were made in the following manner: A fresh cut was made in the loaf and a 5 gm. sample was then cut from the center. This was ground with 25 cc. of distilled water in a small mortar until the bread was well broken. With the exception of the largest pieces, all the finely broken material was poured into the concentration cell.



TABLE II.

*Electrometric and Colorimetric Determination of the Hydrogen Ion Concentration of Bread.*

Baker.	Trade name of bread.	Date of measurement.	pH of bread.	No. of color plate.
		1918		
Hathaway and Sons.	Subway.	Aug. 7	5.01	3
Ward Baking Co.	Mother Hubbard.	" 8	5.03	2
Hathaway.	Subway.	" 7	5.03	3
Ward.	Mother Hubbard.	" 9	5.12	2
"	" "	" 5	5.13	2.5
"	" "	" 7	5.13	3.5
"	Tip Top.	" 7	5.15	3.5
Hathaway.	Subway.	" 7	5.15	4
Ward.	Tip Top.	" 7	5.18	3.5
Hathaway.	Subway.	" 8	5.18	4
Ward.	Oaten Loaf.	" 9	5.20	2.5
"	Daintymaid.	" 7	5.20	3.5
"	Oaten Loaf.	" 8	5.21	3
"	Daintymaid.	" 8	5.21	3
Hathaway.	Vienna.	" 7	5.21	5
Ward.	Daintymaid.	" 6	5.22	3
"	Mother Hubbard.	" 8*	5.23	2.5
Hathaway.	Subway.	" 5	5.23	4.5
Ward.	Tip Top.	" 7	5.23	4
"	Daintymaid.	" 9	5.24	3
"	Mother Hubbard.	" 9*	5.25	3.5
"	Tip Top.	" 8	5.25	4
Hathaway.	Subway.	" 9	5.25	4
General Baking Co.	Mrs. Walker's Prize.	" 8	5.25	4.5
Ward.	Oaten Loaf.	" 8*	5.27	3
"	Tip Top.	" 5	5.27	4
"	Oaten Loaf.	" 9*	5.27	4
Hathaway.	Subway.	" 8*	5.30	4
Ward.	Daintymaid.	" 8*	5.31	3
Hathaway.	Subway.	" 7	5.32	4
General.	Mrs. Walker's.	" 7	5.32	4.5
Hathaway.	Subway.	" 9*	5.32	4.5
General.	Mrs. Walker's.	" 7	5.33	3
"	" "	" 5	5.33	5
Ward.	Daintymaid.	" 6	5.34	3.5
"	Tip Top.	" 8*	5.35	3.5
"	Daintymaid.	" 9*	5.35	3.5
"	"	" 6	5.35	3.5
General.	Mrs. Walker's.	" 9*	5.35	4

TABLE II—*Concluded.*

Baker.	Trade name of bread.	Date of measurement.	pH of bread.	No. of color plate.
		1918		
Ward.	Tip Top.	Aug. 6	5.35	4
General.	Mrs. Walker's.	" 9	5.35	4.5
Hathaway.	Subway.	" 6	5.36	4
Ward.	Daintymaid.	" 6—*	5.40	3
"	Tip Top.	" 9	5.40	4
General.	Mrs. Walker's.	" 5—*	5.42	5
"	" "	" 8*	5.42	5
"	" "	" 6	5.43	5
Ward.	Mother Hubbard.	" 6	5.43	4
Spindler's Bakery.		" 7	5.44	5
Anastos and Chakalis.		" 6	5.47	6
" " "		" 6	5.52	6
Hathaway.	Cream Loaf.	" 7	5.52	5.5
Anastos and Chakalis.		" 6*	5.60	6
Ward.	Tip Top.	" 9*	5.70	5.5

\*Determination made on the day after the loaves were bought and brought into the laboratory.

By comparison with a color chart or with a loaf of bread of known acidity, the hydrogen ion concentration of a loaf of bread may be estimated with an error no greater than that indicated by the above determinations.

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## A QUANTITATIVE STUDY OF THE EVAPORATION OF BLOOD SERUM.

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A need for the dried solids of blood serum in immediately soluble form has led to a study of methods of low temperature evaporation of protein solutions. The method adopted may be of use in other instances.

The blood freshly collected at the abattoir was allowed to clot undisturbed in cylindrical vessels of several gallons capacity. After separating with a thin knife blade the strands of the clot from the sides of the vessel, the serum was syphoned into bottles and promptly centrifuged to remove the small quantity of corpuscles not held by the clot. This obviates laking of the blood, and yields a perfectly clear, amber colored liquid.

The serum containing somewhat less than 9 per cent of total solids, to gain any considerable amount of dried residue, demands the evaporation of a large amount of water. This evaporation may be carried out in a current of warmed air at atmospheric pressure, the exposed surface of the serum being increased by placing the liquid in large, salt-mouth bottles arranged horizontally and rotated. The rate of evaporation so induced is rather low, and this prolonged warming tends to denature the proteins, rendering them less soluble.

The obvious alternative is to carry out the process under reduced pressure. This method was applied with excellent results in the way now to be described, which has the especial advantage that the process may easily be followed in a quantitative manner.



An ordinary distilling flask of at least 1 liter capacity was supplied with perhaps 100 gm. of rather large glass pearls, and weighed. It was then immersed to about the level of the side tube in water of the desired temperature. In our experiments, this temperature did not exceed 50°C. A dropping funnel was introduced into the flask, the end of the stem extending well into the bulb. The side tube of the flask was connected with an efficient condenser, arranged vertically and emptying into a previously weighed bottle of size suitable for trapping the evaporated and recondensed water. After this followed in succession a small, weighed calcium chloride tower, a manometer, and the vacuum pump. The trap bottle and the calcium chloride tower were immersed in ice water, and ice water flowed through the condenser jacket.

The apparatus being evacuated to a pressure of 1 cm. of mercury, or less, the liquid serum was allowed to enter slowly through the funnel, and, as nearly as possible, at the rate at which evaporation proceeded. So treated, the serum foams largely and leaves a friable product that adheres rather loosely to the walls of the flask.

When the desired amount of liquid had been evaporated, the cold water of the condenser jacket was replaced by warm water and air was slowly admitted through the funnel. This was followed by reevacuation. As the evaporation neared completion, the temperature of the bath was raised somewhat. When the serum was dry, the apparatus was disassembled and the parts previously weighed were dried and reweighed.

Through giving the flask a vigorous rotary motion, the pearls were made to loosen and pulverize the product, which was separated from them by pouring onto a sieve of proper mesh.

The following data of preparations show the precision of which the procedure is readily capable. The density of the serum used in the preparations was  $d_4^{25} = 1.0234$ . The bath temperature was between 45 and 50°.

*Quantitative Preparation of Dried Serum.*

Weight of serum delivered to dropping funnel.....	=	102.02
“ “ “ collected in trap bottle.....	=	90.30
“ “ “ “ “ tower.....	=	2.60
“ “ solid residue in flask.....	=	9.11
		<hr/> 102.01

The trapped water was perfectly clear and colorless. Its specific conductance was approximately  $7.7 \times 10^{-5}$ ; it was thus nearly free of electrolytes. It had a slight ammoniacal odor and was faintly alkaline. The pH was 8.1. The pH of the original serum, which had not been adjusted to a particular  $\text{CO}_2$  tension, was 7.65, and its conductance  $1.22 \times 10^{-2}$ . Redissolved in carbon dioxide-free water in its original concentration, the reaction of the preparation was pH 9.4.

The solids of the serum thus constituted 8.93 per cent of the whole. Two different samples of serum from the same source proved closely constant in this respect, as the following data show.

Experiment No.	Liquid serum.	Residue.	Solids in serum.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
770	102.02	9.10	8.92
771	102.02	9.16	8.98
772	102.02	9.11	8.93

The powdered product is of light yellow color and of faint odor. It dissolves readily in distilled water to a solution that is but slightly turbid. Determination of its nitrogen content by the Kjeldahl method gave the following results:

*Nitrogen.*  
*per cent*

11.66

11.83

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Average, 11.75

According to Michaelis and Davidsohn, the isoelectric point of serum albumin appears at about pH 4.7. The electrometric titration of serum as reported by McClendon and confirmed by determinations in this laboratory shows that when approximately 0.06 cc. of normal hydrochloric acid is added to each cc. of serum, the pH is 4.5. Serum so treated and evaporated leaves, as would be expected, a product more friable and less soluble in water than that from the untreated serum. Such a preparation is presumably near the isoelectric point of the proteins, and readily dissolves upon the addition of acid or of alkali.

Data of a preparation from serum treated with less acid than the above requirement are as follows:

*Preparation of Acidified Serum.*

	gm.
Weight of serum taken.....	102.02
“ “ normal hydrochloric acid added.....	4.08
	<hr/>
	106.10
Weight of liquid water collected.....	78.88
“ “ water in drying tower.....	18.03
“ “ solid residue.....	9.13
	<hr/>
	106.04

Before evaporation, the reaction of the acidified serum unadjusted to a definite carbon dioxide tension was pH 5.3. The redissolved preparation was slightly less acid, pH 5.5. In its behavior it lies between the preparation from unacidified serum and that from serum evaporated at its isoelectric point. It has the advantage of being more easily soluble than the latter, and, for some purposes, of having a less alkaline reaction than the former. The carbonates of the serum can thus conveniently be transformed into chlorides or into the salts of other acids.

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